

Imaging Biomarkers for Oligometastatic Colorectal Cancer



Jamie Franklin

Oriel College

University of Oxford

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This thesis is dedicated to:

Niki, Samuel and Beatrix Franklin

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Summary of Work

Work completed

I obtained funding from the Oxford Health Sciences Research Committee to perform the prospective FILM trial, studying colorectal liver metastases (see Appendix 7). I designed the trial protocol and gained ethical approval for the study, and subsequently prepared and submitted a substantial amendment (see Appendix 8) on behalf of the PI, Professor Fergus Gleeson. I was subsequently awarded a CRUK Small Projects Grant to perform the genomic analyses (see Appendix 7).

I recruited patients to the prospective component of this study, performing or supervising all components of the imaging and histopathology acquisition and analysis. I worked with colleagues in the Institute of Biomedical Engineering to analyse the DCE-MRI data, including providing input into engineering projects to improve motion correction and develop a subregion analysis tool, which were then applied to the clinical data. Work arising from these collaborations has been presented at MICCAI and ECR, and submitted for publication in *European Radiology*. I collated a retrospective cohort of imaging and histopathology of resected CRLM, and investigated histopathological heterogeneity and conventional and novel ^{18}F -FDG-PET imaging biomarkers in the cohort.

In the Sharma Lab in the Department of Oncology, I learnt 2D and 3D cell culture techniques, and used these to investigate a panel of CRC cell lines for variations in IHC expression and glucose uptake.

Collaborating with the Tomlinson Lab in the Wellcome Trust Centre for Human Genetics, I learnt techniques for extracting and quantifying DNA from FFPE specimens, which I then applied to CRLM, to extract DNA from metastases and subregions of metastases with different histopathological and imaging features.

I was first-author on an article published in *Lancet Oncology* discussing imaging biomarkers in oligometastatic cancer. I co-authored a further review article discussing biomarkers in CRLM, published in the *European Journal of Surgical Oncology*.

Support received

Histopathology - I received training and support in performing the IHC staining from Leticia Campo in the GCP laboratory, and from Marcus Green in producing and imaging FFPE slides. Two consultant histopathologists, Professor Rob Goldin and Dr Lai Mun Wang, helped me to develop the methodology, and perform the scoring of the histopathological and IHC features of CRLM.

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Publications

The following publications are directly related to work presented in this thesis:

1. Papież BW, **Franklin JM**, Heinrich MP, Gleeson FV, Brady M, Schnabel JA. GIFTed Demons: deformable image registration with local structure-preserving regularization using supervoxels for liver applications. *J Med Imag.* 2018 Oct 28;5(02):1–14.
2. Jones RP, Brudvik KW, **Franklin JM**, Poston GJ. Precision surgery for colorectal liver metastases: Opportunities and challenges of omics-based decision making. *Eur J Surg Oncol.* 2017 May;43(5):875–83.
3. **Franklin JM**, Sharma RA, Harris AL, Gleeson FV. Imaging oligometastatic cancer before local treatment. *Lancet Oncol.* 2016 Sep;17(9):e406–14.
4. Irving B, **Franklin JM**, Papież BW, Anderson EM, Sharma RA, Gleeson FV, et al. Pieces-of-parts for supervoxel segmentation with global context: Application to DCE-MRI tumour delineation. *Med Image Anal.* 2016 Aug;32:69–83.

The following publications on related work were published based on work undertaken during the period of study covered by this DPhil:

1. Findlay JM, Bradley KM, Wang LM, **Franklin JM**, Teoh EJ, Gleeson FV, et al. Metabolic nodal response as a prognostic marker after neoadjuvant therapy for oesophageal cancer. *Br J Surg.* 2017 Mar;104(4):408–17.
2. Findlay JM, Bradley KM, Wang LM, **Franklin JM**, Teoh EJ, Gleeson FV, et al. Predicting Pathologic Response of Esophageal Cancer to Neoadjuvant Chemotherapy: The Implications of Metabolic Nodal Response for Personalized Therapy. *J Nucl Med.* 2017 Feb;58(2):266–75.
3. Hill EJ, Roberts C, **Franklin JM**, Enescu M, West N, MacGregor TP, et al. Clinical Trial of Oral Nelfinavir before and during Radiation Therapy for Advanced Rectal Cancer. *Clin Cancer Res.* 2016 Apr 14;22(8):1922–31.

4. **Franklin JM**, Gebiski V, Poston GJ, Sharma RA. Clinical trials of interventional oncology-moving from efficacy to outcomes. *Nat Rev Clin Oncol*. 2015 Feb; 12(2):93–104.
5. Parvizi N, **Franklin JM**, McGowan DR, Teoh EJ, Bradley KM, Gleeson FV. Does a novel penalized likelihood reconstruction of 18F-FDG PET-CT improve signal-to-background in colorectal liver metastases? *European Journal of Radiology*. 2015 Oct;84(10):1873–8.

Presentations

The following presentations are directly related to work presented in this thesis:

1. **Franklin JM**, Irving B, Papiez BW, Kallehauge J, Anderson EM, Wang L, Goldin R, Brady M, Gleeson FV. Tumour subregion analysis of colorectal liver metastases using DCE-MRI: comparison with histological subregions and impact on PK analysis. *Insights Imaging* (2017) (Suppl 1):S1–S583.
2. Papież BW, **Franklin JM**, Heinrich MP, Gleeson FV, Schnabel JA. Liver Motion Estimation via Locally Adaptive Over-Segmentation Regularization. In: *Medical Image Computing and Computer-Assisted Intervention – MICCAI 2015*. Cham: Springer, Cham; 2015. pp. 427–34. (Lecture Notes in Computer Science; vol. 9351).

In addition, invited presentations on components of this DPhil were made at the 2nd Colorectal Cancer Network Meeting in March 2015, and at the Oxford Institute of Radiation Oncology in September 2015.

The following presentations were based on work completed during the period of study covered by this DPhil:

1. **Franklin JM**, Syed, N, Macintyre A, Gleeson FV, Anderson EM. Whole-body MRI for recurrent colorectal cancer – feasibility and initial experience. NCRI, Liverpool, UK, 2015.
2. Findlay JM, **Franklin JM**, Teoh E *et al*. Baseline 18F-FDG-PET/CT is associated with progression to metastatic disease during neoadjuvant chemotherapy for oesophageal cancer. EANM Annual Meeting, Gothenburg, Sweden, 2014.

Abstract

Imaging Biomarkers for Oligometastatic Colorectal Cancer

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Oligometastatic colorectal cancer represents an intermediate disease state potentially treatable with curative intent. Novel treatment strategies are expanding the opportunity to offer local treatment for limited metastatic disease. It is therefore increasingly important to define patients likely to benefit from these targeted treatments. Diagnostic imaging is central to patient management, to establish the burden and distribution of metastatic disease. This thesis explores the potential value of novel imaging biomarkers in patients with colorectal liver metastases (CRLM). In a prospective study, perfusion CT (pCT) and dynamic contrast-enhanced MRI (DCE-MRI) were performed prior to resection, and a retrospective series of patients with resected CRLM was collated. Resected metastases were analysed for relevant morphological features and IHC expression. Novel and conventional imaging variables were derived based on CT, MRI, pCT, DCE-MRI and ^{18}F -fluorodeoxyglucose positron emission tomography (^{18}F -FDG-PET), and their associations with histopathological features, immunohistochemical expression and patient outcomes analysed. A novel subregion analysis based on DCE-MRI data was developed to classify tumour subregions, using a histological reference standard. The results presented in this thesis confirm that the number and distribution of hepatic metastases are prognostic markers for patients with resected CRLM, and that higher SUVmean of liver metastases, and higher SUVmax of the background liver, are potential prognostic biomarkers. Histopathological assessment of resected CRLM highlighted that there is significant between and within patient variability of clinically relevant histopathological features and IHC expression, which would support the potential utility of imaging biomarkers to characterise multi-site disease. Analysis of functional imaging data demonstrated positive correlations between imaging variables and clinically relevant markers of tumour vascularity and metabolism, while subregion analysis of DCE-MRI data produced good spatial correlation with viable and non-viable tumour based on histopathology, with significant differences in derived pharmacokinetic parameters between subregions.

Contents

Introduction	1
1. Colorectal cancer	1
2. Oligometastases	2
3. Prognostic models for oligometastatic CRLM	4
4. Imaging for oligometastatic CRLM	7
5. Novel imaging biomarkers for oligometastatic CRLM	9
6. Challenges	21
Aims and objectives	24
Materials and Methods	25
1. Functional Imaging of Liver Metastases (FILM) study	25
2. Imaging acquisition	31
3. Imaging analysis	34
4. Histopathological Analysis	40
Prognostic imaging biomarkers in oligometastatic CRLM	46
1. Introduction	46
2. Chapter Methods	50
3. Results	54
4. Discussion	63
Histopathological heterogeneity of CRLM	69
1. Introduction	69
2. Chapter Methods	71
3. Results	82
Discussion	96

Morphological and functional imaging of CRLM	104
1. Introduction	104
2. Chapter Methods	106
3. Results	114
4. Discussion	131
Discussion	143
Future directions for research	156
Appendix 1: Imaging and histology	159
Appendix 2: Individual patient variables	161
Appendix 3: pCT protocol	163
Appendix 4: DCE-MRI protocol	165
Appendix 5: DCE-MRI timings	168
Appendix 6: Pathological assessment	169
Appendix 7: Funding Letters	170
Appendix 8: Ethical Application and Approval	173
References	175

Glossary

Abbreviation	Meaning
¹⁸ F-FDG	¹⁸ F-fluorodeoxyglucose
2-DG	2-deoxy-D-Glucose
2-DG6P	2-deoxy-D-Glucose-6-phosphate
2D	Two-dimensional
3D	Three-dimensional
ADC	Apparent Diffusion Coefficient
AI	Artificial Intelligence
AIF	Arterial Input Function
ATP	Adenosine Triphosphate
BF	Blood Flow
BRAF	Gene responsible for coding the B-Raf protein
BSA	Bovine Serum Albumin
BV	Blood Volume
CA9/CAIX	Carbonic Anhydrase 9
CD105	Cluster of Differentiation 105
CD31	Cluster of Differentiation 31
CD34	Cluster of Differentiation 34
CEA	Carcinoembryonic Antigen
ceCT	Contrast-Enhanced CT
CN	Copy Number
CNV	Copy Number Variation
CRC	Colorectal Cancer
CRF	Clinical Research Fellow
CRLM	Colorectal Liver Metastases
CRP	C-Reactive Protein
CRUK	Cancer Research UK
CT	Computed Tomography
CT CAP	Chest, Abdomen and Pelvis CT
DCE-MRI	Dynamic Contrast Enhanced MRI
DFI	Disease-Free Interval
DICOM	Digital Imaging and Communications in Medicine
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSC	Dice Similarity Coefficient
DWI	Diffusion Weighted Imaging
EDTA	Ethylenediaminetetraacetic acid
EF	Enhancing Fraction - check used

EHD	Extrahepatic Disease
EMVI	Extramural Venous Invasion
ERS	Epitope Retrieval Solution
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FILM	Functional Imaging of Liver Metastases (Study)
FOLFOX	Combination FOLinic acid, Fluorouracil and OXaliplatin chemotherapy
FSE	Fast Spin Echo
fsFRFSE	Fast Recovery Fast Spin Echo sequence (with fat suppression)
GLUT	Glucose Transporter
H&E	Haematoxylin and Eosin
HAF	Hepatic Arterial Fraction
HCC	Hepatocellular Carcinoma
HIER	Heat-Induced Epitope Retrieval
HIF	Hypoxia Inducible Factor
HPB	Hepatopancreaticobiliary
hPFS	Hepatic Progression-free Survival
Hu	Hounsfield units
IBME	Institute of Biomedical Engineering
IHC	Immunohistochemistry or Immunohistochemical
IV	Intravenous
KRAS	Gene responsible for coding the K-Ras protein
KRPH	Krebs-Ringer-Phosphate-Hepes buffer
kV	Kilovolts - check used
LAVA	Liver Acquisition with Volume Acquisition
LOH	Loss of Heterozygosity
MAPD	Median Absolute Pairwise Difference
mCRC	Metastatic CRC
MDT	Multidisciplinary Team
MIP	Molecular Inversion Probe
mRECIST	Modified RECIST
mrEMVI	Extramural Venous Invasion evaluated by MRI
MRI/MR	Magnetic Resonance Imaging
MTT	Mean Transit Time
MTV	Metabolic Tumour Volume
MVD	Microvessel Density
NAPD	Nicotinamide adenine dinucleotide phosphate
ndSNPQC	Normal Diploid SNP Quality Control
NEAA	Non-Essential Amino Acids
NIHR	National Institute for Health Research
NLR	Neutrophil to Lymphocyte Ratio
NPV	Negative Predictive Factor

NSCLC	Non-small Cell Lung Cancer
OS	Overall Survival
OSEM	Ordered Subset Expectation Maximisation
OUH	Oxford University Hospitals NHS Foundation Trust
PCA	Principle Component Analysis
pCT	Perfusion CT
PE	Peak Enhancement
PET	Positron Emission Tomography
PFS	Progression-free Survival
PK	Pharmacokinetic
PLR	Platelet to Lymphocyte Ratio
PPV	Positive Predictive Factor
PVIF	Portal Venous Input Function
QC	Quality Control
R1 resection	Positive for microscopic tumour at the resection margin
R2 resection	Positive for macroscopic tumour at the resection margin
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
RECIST	Response Evaluation Criteria in Solid Tumours
RNA	Ribonucleic Acid
ROI	Region of Interest
SABR	Stereotactic Ablative Radiotherapy (see also SBRT)
SAP	Surface Area Product - checked used
SBRT	Stereotactic Body Radiation Therapy (see also SABR)
SCNA	Somatic Copy Number Aberration
SGLT	Sodium-Dependent Glucose Transporters
SIRT	Selective Internal Radiotherapy
SNP	Single Nucleotide Polypeptides
SSFSE	Single Shot Fast Spin Echo
SUV	Standardised Uptake Value
T1W	T1-weighted
T2W	T2-weighted
TACE	Transcatheter arterial chemoembolization
TE	Echo Time
TGV	Tumour Glycolytic Volume
TLG	Total Lesion Glycolysis
ToF	Time of Flight
TR	Repetition Time
TRG	Tumour Regression Grading
VEGF	Vascular Endothelial Growth Factor
VOI	Volume of Interest
WCC	White Cell Count

List of Tables

Chapter 1 - Introduction

Table 1a: Prognostic factors included in prognostic models following hepatic metastasectomy in patients with CRLM

Table 1b: Occurrence of prognostic imaging biomarkers for resected CRLM

Chapter 2 - Materials and Methods

Table 2a: Summary of demographic and clinical parameters recorded for patients in the prospective and retrospective studies

Table 2b: Summary characteristics of prospective FILM study patients (n=14)

Table 2c: Summary of the prospective FILM study patients (n=14)

Table 2d: Summary characteristics of patients in the retrospective FILM study (n= 96)

Table 2e: Summary of extracted ^{18}F -FDG-PET/CT parameters

Table 2f: Summary of extracted pCT parameters

Table 2g: Summary of extracted DCE-MRI parameters

Chapter 3 - Prognostic imaging biomarkers in oligometastatic CRLM

Table 3a: Summary characteristics of patients in the retrospective FILM study

Table 3b: Summary of conventional imaging biomarkers n the retrospective FILM study

Table 3c: Summary of ^{18}F -FDG-PET parameters

Table 3d: Univariate analysis of variables for DFS and hPFS

Table 3e: Multivariate model of variables for DFS and hPFS

Chapter 4 - Histopathological heterogeneity of CRLM

Table 4a: Histopathological features and IHC staining of CRLM

Table 4b: Patterns of tumour-liver interface in individual metastases

Table 4c: IHC expression within and between patients with CRLM

Table 4d: IHC staining of cell pellets of colorectal cell lines (% staining cells)

Table 4e: Glucose consumption and uptake experiments

Table 4f: DNA yield, total copy number aberrations, percentage loss of heterozygosity and QC metrics for samples analysed by OncoScan.

Chapter 5 - Morphological and functional imaging of CRLM

Table 5a: Diagnostic accuracy of MRI for the presence of encapsulation of CRLM metastases.

Table 5b: DSC comparing ROIs derived from imaging and histopathology

Table 5c: Proportion of viable tumour by visual assessment, and calculated from the histopathology-defined and imaging-derived subregions

Table 5d: FILM Study functional imaging parameters

Table 5e: Prospective FILM study selected histological and IHC phenotypes

Table 5f: SUVmax compared with histological features and IHC

Appendices

Table A1a: Imaging and histology available in the retrospective cohort

Table A2a: Selected patient variables used in the retrospective cohort study

Table A5a: DCE-MRI timings for the FILM study

List of Figures

Chapter 3 - Prognostic imaging biomarkers in oligometastatic CRLM

Figure 3a: Kaplan-Meier curves for DFS and hPFS for the entire cohort

Figure 3b: Histogram of sizes of largest metastases

Figure 3c: Correlation matrix of ^{18}F -FDG-PET imaging variables

Figure 3d: Kaplan-Meier curves of DFS for patients with resected CRLM grouped by selected clinical and radiological variables

Figure 3e: Kaplan-Meier curves of hPFS for patients with resected CRLM grouped by selected clinical and radiological variables

Chapter 4 - Histopathological heterogeneity of CRLM

Figure 4a: Clonal evolution on MRI after initial response to chemotherapy

Figure 4b: Tumour masks for masks for FFPE macrodissection

Figure 4c: GLUT1 and ki67 staining of SW1222 and HT29 spheroids

Chapter 5 - Morphological and functional imaging of CRLM

Figure 5a: Tumour subregion delineation on H&E stained histopathology slide

Figure 5b: Two-stage semi-automated segmentation of manually delineated liver metastasis by PCA clustering based on DCE-MRI signal-time curves

Figure 5c: Dice similarity coefficients of between tumour subregions defined by imaging and histopathology

Figure 5d: Mean K^{trans} of CRLM and of image-derived tumour (purple) and non-tumour subregions

Figure 5e: Mean k_{ep} of CRLM and of image-derived tumour and non-tumour subregions

Figure 5f: Subregion analysis of K^{trans} parameter maps

Figure 5g: Blood volume compared with K^{trans}

Figure 5h: Blood volume compared with k_{ep}

Figure 5i: SUVmax compared with K^{trans}

Figure 5j: SUVmax compared with k_{ep}

Figure 5k: k_{ep} compared with CA9

Figure 5l: Blood volume compared with microvessel density

Figure 5m: Blood flow compared with microvessel density

Figure 5n: SUVmax compared with GLUT1 expression Chapter 6

Chapter 1

Introduction

1. *Colorectal cancer*

Each year approximately 40,000 patients in the UK (CRUK Bowel Cancer Statistics, 2015), and approximately 1 million worldwide (Ruers et al., 2012), are diagnosed with colorectal cancer (CRC). It is the second most common cancer in the UK in terms of both incidence and mortality (CRUK Bowel Cancer Statistics, 2015). There has been significant progress in the prognosis for patients diagnosed with CRC, but despite this, 50% and 10-15% of patients diagnosed will develop liver and lung metastases respectively (Ruers et al., 2012, Salah et al., 2012). 90% of tumour deaths are due to metastatic disease, and effective treatment of metastatic disease remains a major challenge.

2. *Oligometastases*

The majority of patients who develop metastatic disease are treated with systemic chemotherapy with palliative intent. However, metastatic disease behaviour and patient prognosis is non-uniform. In certain tumour types and metastatic locations, such as the liver (Hughes et al., 1986, Nordlinger et al., 1996, Fong et al., 1999, Rees et al., 2008), lung (Pastorino et al., 1997) and adrenal glands (Strong et al., 2007), local control of metastatic disease can result in long-term disease free survival. This observation led to the hypothesis that, in some tumour types, there exists an intermediate state of clinically overt metastatic disease, which remains amenable to curative treatment. This has been termed 'oligometastatic disease' (Hellman & Weischelbaum, 1995).

There is growing academic interest in oligometastases (Franklin et al., 2016), in parallel with an expansion in clinical intervention in this setting. In the UK and US: the hepatectomy rate in UK patients with colorectal liver metastases (CRLM) increased from 1.7% in 1998 to 3.8% in 2004, but with significant variation from region-to-region (Morris et al., 2010), while US data shows rises in the most common metastasectomy procedures across several cancer types (Bartlett et al., 2015). While surgical metastasectomy remains the dominant treatment modality for patients considered oligometastatic, there is also greater acceptance and adoption of local ablative techniques such as radiofrequency ablation, microwave ablation and stereotactic radiotherapy (SABR/SBRT). These techniques,

particularly SBRT, allow multiple sites to be treated, and are typically less morbid than surgical metastasectomy (Tree et al., 2013). Oncologists are now offering locally-directed therapy in most common tumour types and at the majority of disease sites (Dagan et al., 2016; Franklin et al., 2016). There are numerous ongoing trials evaluating SBRT and radiotherapy for treating oligometastases (Reyes et al., 2015).

As these treatment modalities have gained wider acceptance in clinical practice, advances in surgical practice have broadened the criteria for resectability (Morris-Stiff et al., 2016), and local ablative techniques are now routinely considered as part of a multimodal treatment strategy, meaning more extensive oligometastatic disease can be treated. For patients with resectable liver metastases from CRC, more effective neoadjuvant chemotherapy has also improved the PFS (Nordlinger et al., 2013), while 'conversion' chemotherapy can render patients with initially unresectable disease operable (Adam et al., 2009). It is also recognised that patients with multi-organ metastatic disease can achieve long-term survival if treated aggressively (Adams et al., 2003; Dave et al., 2014), providing clinical justification for combined treatment approaches. There has also been a fall in perioperative morbidity (Bartlett et al., 2015), which may lower the threshold for considering metastasectomy based on patient factors.

Although the "therapeutic opportunity" (Treasure, 2012) presented by limited metastatic disease is growing, the oligometastatic state remains undefined for the

majority of cancers and, even for established settings, clinical practice continues to evolve. Labelling patients as oligometastatic provides an opportunity for aggressive radical treatment but, without evidence for efficacy from randomised controlled trials, this could expose patients to futile therapies with their associated costs, morbidity and mortality, without clinical benefit, and burden health systems with additional treatments that may not be clinically or cost-effective. Despite apparent local disease control, the majority of patients will suffer further metastatic relapse. Of patients with CRC undergoing liver metastasectomy, only 38-58% of patients will survive to 5 years (Spelt et al., 2012) and a significant minority will suffer relapse within the first year of surgery (Gomez et al., 2010). Although it is now possible to treat multisite metastatic disease, we are not able to reliably determine beforehand which patients will benefit from aggressive local treatment strategies. In the setting of widening clinical opportunities for intervention, robust and consistent work-up is crucial, and developing novel prognostic biomarkers a priority.

3. *Prognostic models for oligometastatic CRLM*

Several authors have analysed retrospect cohorts of patients who have undergone liver metastasectomy for CRC to establish which clinical factors are prognostically relevant in this setting. Based on some of the larger series, prognostic models have been developed (Table 1a). There is significant overlap in the factors

analysed in these studies, and these have been subject to systemic review and meta-analysis (Spelt et al., 2012; Kanas et al., 2012).

Table 1a: Prognostic factors in prognostic models following liver metastasectomy for CRC

Factors	Prognostic model			
	Nordlinger et al. (1996)	Fong et al. (1999)	Iwatsuki et al. (1999)	Rees et al. (2008)
Patients, n	1568	1001	305	1005
Age	+	-	-	-
Primary serosal invasion	+	NA	NA	NA
Primary nodal stage	+	+	-	+
Primary differentiation	NA	NA	-	+
DFI from primary	+	+	+	NA
Size	+	+	+	+
Number	-	+	+	+
Bilobar metastases	-	-	+	-
EHD	-	-	+	+
Preoperative CEA	-	+	NA	+
Surgical margin	+	-	+	+
Notes DFI = disease free interval from resection of the primary tumour to metastatic recurrence. Number = number of liver metastases. Bilobar = metastases in both the left and right lobe of the liver. Size = size of the largest metastasis. EHD = extrahepatic disease. CEA = carcinoembryonic antigen. + = factor identified as predictive. - = factor identified as not predictive. NA = factor not analysed.				

However, prognostic biomarkers derived from retrospective series predate modern therapeutic options, and appear not to appropriately stratify patients for treatment (Ayez et al., 2011; Roberts et al., 2014). A challenge for the clinical

application of these prognostic models is that there has never been a randomised controlled trial (RCT) of metastasectomy for CRLM, and therefore there is no strong evidence that patients with a particular risk profile based on these prognostic factors derive benefit from intervention. As a result, there are no hard selection criteria for considering radical therapy. Although there is clearly benefit from intervening in higher risk patients, the threshold at which this becomes clinically- or cost-effective is unknown. Nor are there randomised populations in whom potential methods of risk stratification could be tested for improvement; all the patients in the retrospective cohort studies (Tables 1 a and 1b) underwent liver resection. A single randomised study in 119 patients has been performed of RFA and systemic therapy compared with systemic therapy alone in unresectable CRLM, which demonstrated no differences in median progression-free survival (PFS) or overall survival (OS) between the groups (Ruers et al., 2012). This contrasts with the evidence for local treatment of cerebral oligometastases, which has been investigated in several RCTs (summarised by Scoccianti and Ricardi, 2011) and the selection criteria for these trials have been used to dictate clinical guidelines (e.g. Soffietti et al., 2017). Local treatment of CRLM lacks this supportive evidence, and the prognostic factors are, at best, guidelines to inform clinical decision-making.

4. *Imaging for oligometastatic CRLM*

Imaging already plays a pivotal role in describing the total metastatic disease burden in patients with oligometastatic CRC. Most pre-operative imaging in patients with CRLM is focused on demonstrating the hepatic disease burden, which informs the local treatment options, and detecting extrahepatic disease. Much of the imaging literature around colorectal metastases has focused on these important determinants of treatment. Computed tomography (CT) is usually the first staging investigation in patients with CRC, and during follow-up. However, sensitivity of contrast-enhanced CT for colorectal metastatic disease in the liver is approximately 85% on a per-patient basis, falling to 74% on a per-lesion basis (Niekel et al., 2010). This is inadequate in the radical treatment setting and therefore liver-specific imaging is routinely recommended. Multiparametric liver MRI, incorporating multiphase post-contrast MRI using hepatocyte specific contrast media and diffusion-weighted imaging (DWI) has become established as the technique with the highest per-lesion sensitivity for CRLM (Muhi et al., 2010; Hekimoglu et al., 2011). ^{18}F -fluorodeoxyglucose position emission tomography (^{18}F -FDG-PET) CT is used in combination with CT (^{18}F -FDG-PET/CT) in many settings to stage patients with suspected oligometastatic cancer. In patients with CRLM, it detects additional extrahepatic disease in a significant minority of patients (Maffione et al., 2015) leading to improved selection for radical treatment. Patients undergoing hepatectomy for CRLM staged with ^{18}F -FDG-PET/CT have improved survival compared with historical data in patients not

investigated by ^{18}F -FDG-PET or ^{18}F -FDG-PET/CT (Fernandez et al., 2004) and undergo fewer futile laparotomies (Ruers et al., 2009).

A higher pre-operative disease burden is associated with worse outcome: the number, size and distribution of metastases have been found to be prognostically relevant in patients with CRC undergoing liver metastasectomy (Table 1b) (Spelt et al., 2012; Kanas et al., 2012). For local ablative treatments, there is now emerging evidence that similar markers of disease burden are prognostically relevant (Shady et al., 2016). Treatment decisions are informed by these criteria: patients with poor prognostic factors may receive neoadjuvant chemotherapy prior to resection, or may be considered unsuitable for radical treatment.

Table 1b: Occurrence of prognostic imaging features for resected colorectal liver metastases*

	Nordlinger et al (1996)	Rees et al (2008)	Fong et al (1999)	Malik et al (2007)	Zakaria et al (2007)	Yamaguchi et al (2008)	Minagawa et al (2007)	Iwatsuki et al (1999)	Tan et al (2008)	Schindl et al (2005)	Konopke et al (2009)	Tanaka et al (2004)	Lise et al (2001)	Ueno et al (2000)	Nagashima et al (2006)
Patients, n	1568	1005	1001	687	662	380	369	305	285	269	201	149	135	85	81
Factors															
• Number	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+
• Size	+	+	+	-	-	+	-	+	-	-	-	-	-	-	+
• Bilobar	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
• EHD	NA	+	+	NA	NA	+	-	+	NA	NA	NA	-	NA	NA	+

Notes

*adapted from the review by Spelt and colleagues (2012)

Number = number of liver metastases. Bilobar = metastases in both the left and right lobe of the liver. Size = size of the largest metastasis. EHD = extrahepatic disease. + = factor identified as predictive. - = factor identified as not predictive. NA = factor not analysed.

However, prognostic scores based on disease burden biomarkers were not developed in the setting of consistent, modern imaging. More comprehensive imaging strategies will detect additional disease that would have remained occult

in the series on which prognostic scores were based. The prognostic scoring systems also predate effective neoadjuvant chemotherapy. Both radiological and pathological response to chemotherapy are indicators of good prognosis (Muhi et al., 2010), whereas progression through treatment is associated with a poor prognosis (Adam et al., 2004). The majority of clinical scoring systems were derived using patients staged with historical imaging strategies without incorporating treatment response in the analysis. This perhaps helps explain why these tools are relatively poor discriminators (Roberts et al., 2014).

This thesis focuses on the potential to develop novel imaging biomarkers for patients with colorectal oligometastases.

5. *Novel imaging biomarkers for oligometastatic CRLM*

A biomarker is a “defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention, including therapeutic interventions” (Biomarkers Definitions Working Group, 2001). Some definitions have not considered imaging data to represent biomarkers, restricting their definition to molecular biomarkers, but a wider definition which incorporates imaging is now generally accepted (O’Connor et al., 2016). Existing clinical and imaging biomarkers for oligometastatic CRC have been discussed above in sections 1.3 and 1.4. These have mainly focused on

prognostic biomarkers; an attempt to establish *who* should undergo treatment, particularly surgical metastasectomy.

A recent 'roadmap' for imaging biomarkers (O'Connor et al., 2016) has set out the various steps in imaging biomarker discovery and validation, and documents their widespread use in current clinical practice, their key attributes and gives an overview of the scale of the scientific effort to develop imaging biomarkers. While the imaging assessment of disease extent and burden has been repeatedly demonstrated to be prognostically relevant, there is scope for imaging to further characterise tumour phenotypes and therefore provide novel biomarkers, which may be prognostically or therapeutically relevant. Conventional CT and MRI demonstrates variation in tumour morphology (Namasivayam, Martin & Saini, 2007) and in response to chemotherapy (van Kessel et al., 2013), while novel imaging techniques can quantify aspects of tumour morphology and physiology that reflect differences in tumour biology. The subsequent sections of this thesis discuss how novel image analysis and imaging techniques could be used to better define the tumour phenotypes of oligometastatic CRLM, firstly by morphological imaging (tumour size and structure), and secondly by functional tumour imaging (tumour physiology).

Morphological tumour imaging

Tumour response to neoadjuvant chemotherapy

Patients with oligometastatic CRLM are often offered neoadjuvant chemotherapy as the first-line treatment, either due to anticipated poor tumour biology, or in an attempt to render bulky disease surgically resectable. As with treatment of the primary malignancy, pathological tumour response to chemotherapy is associated with improved prognosis (Vigano et al., 2013). Usually radiological response is determined by an objective solid tumour evaluation criteria such as Response Evaluation Criteria in Solid Tumours criteria (RECIST; Eisenhauer et al., 2009), based on CT or MRI. At this time, the preferred methodology for using treatment response as a prognostic biomarker for patients with oligometastatic CRLM has not been established, and response as a prognostic biomarker has not been incorporated into existing prognostic models (Table 1a). Notwithstanding this fact, progressive disease through chemotherapy is a poor prognostic marker and failure to achieve disease control with chemotherapy is usually considered a contraindication to a radical treatment strategy (Adam et al., 2004).

Analysis of morphological features

The pathological literature describes the varied histopathological features of CRLM, both their internal architecture and their interface with the surrounding liver parenchyma (Knijn et al., 2013, Vermeulen et al., 2001). Several series have examined the post-surgical outcomes in patients with these features (Knijn et al., 2013). These features are heterogeneous, varying both within tumours and

between metastases even within individual patients (Eefsen et al., 2012). However, these can usually only be demonstrated following resection, and are therefore of limited value when planning treatment.

The macroscopic histopathological structure of tumours gives rise to the imaging phenotype of conventional imaging. Where a robust association between imaging and histopathological features is demonstrated, imaging can be used as an *in vivo*, non-invasive surrogate for histopathological assessment. This strategy has been used successfully in cancer imaging for validating T- and N-staging in many primary tumour types, and, more recently, for the development and subsequent validation of the MRI feature extramural vascular invasion (mrEMVI) in rectal cancer (Brown et al., 2003). Histopathological EMVI was already a recognised histopathological indicator of poor prognosis, and the MRI feature has subsequently been validated as a prognostic biomarker in the clinic (Hunter et al., 2011; Siddiqui et al., 2017), and is used to stratify patients to different treatments (Chand et al., 2014). Modern CT and MRI imaging systems incorporating respiratory gating can image the liver at high spatial resolution and have the potential to characterise clinically relevant histopathological morphological features of CRLM.

Observed semantic morphological imaging features, like EMVI, can be assessed subjectively, to produce useful categorical classifications. Although subjective assessment can introduce inter- and intra-observer variation, careful validation can

ensure reproducibility, and these semantic features are more readily applicable across imaging platforms than quantitative imaging biomarkers. In contrast, novel imaging analysis techniques, typically but not necessarily based on cross-sectional imaging such as CT or MRI, can mathematically describe a much greater number of quantitative image properties that underlie visual features (e.g. Depeursinge et al., 2014). These are attractive as they can be applied to large (retrospective) datasets and, once the methodology is established, do not suffer from inter-observer variation. Attempts have been made to prognosticate for patients with CRC based on texture profile of the primary tumour (Ng et al., 2013) and background liver parenchyma (Miles et al., 2009).

Internal composition, tumour viability and response to treatment

There has been relatively little research on using imaging to establish the internal composition of liver metastases, as the imaging literature focuses predominantly on lesion detection and classification. Histopathologically, liver metastases are variably composed of tumour cells, fibrosis, necrosis, mucin, and may contain areas of calcification, particularly following treatment. The internal composition of tumours varies from lesion-to-lesion, and is also affected by adjuvant treatment (Rubbia-Brandt et al., 2007). A few studies have used MRI to attempt to delineate the internal composition of tumours, some using spatially co-registered imaging. Viable tumour is typically low T1 signal compared with background liver, intermediate-high T2 signal at MRI (Outwater et al., 1991; Milot et al., 2010). Following contrast enhancement, cellular tumour generally shows early

enhancement with rapid washout (Dao et al., 1993; Koh et al., 2014), although the data on liver metastases is largely from contrast-enhanced ultrasound (e.g. Kong et al., 2016), since the fixed time points of standard post-contrast liver MRI may miss early enhancement, while contrast-enhanced ultrasound provides higher temporal resolution imaging.

CRLM often exhibit classical garland necrosis: areas of necrotic debris, sometimes referred to as 'dirty', 'usual' or intra-acinar necrosis, surrounded rim of viable tumour (Wyatt, Hubscher and Goldin, 2012). On imaging, necrotic material has been described as hyperintense on T1-weighted imaging and typically low (but variable) signal on T2-weighted imaging (Outwater et al 1991, Milot et al., 2010). Following gadolinium-based IV contrast, necrotic material shows late enhancement (Koh et al., 2014). Intratumoural fibrosis is described as low signal on T1-weighted imaging (Outwater et al 1991, Milot et al., 2010) and has been found to be high T2 signal relative to liver (Milot et al., 2010), although the peripheral dense fibrotic pseudocapsule has been reported to be low T2 signal (Outwater et al., 1991; Fibrosis also typically demonstrates late accumulation of IV contrast (Mittal et al., 2015; Disertori et al., 2016). Mucin is a relatively uncommon feature of CRLM but, similarly to primary CRC, can be observed as pools of high T2 signal (Kim et al., 2003) on MRI. Calcification causes signal voids on both T1 and T2-weighted MRI.

Based on differential MRI features of intratumoral components, it may be possible to perform in vivo assessments of the proportion of viable tumour (Franklin et al., 2017) with CRLM, both before and following treatment with chemotherapy. The proportion of viable and necrotic tumour at baseline is generally considered to occur typically as a result of intra-lesional hypoxia and ischaemia as tumour growth outstrips angiogenesis or vascular cooption, and has not been found to be prognostically relevant (Serrablo et al., 2016).

Tumour response to chemotherapy is characterised histologically by reduced viable carcinoma associated with intratumoural fibrosis, which generally predominates as a treatment response over intratumoural necrosis (Poultsides et al., 2012). The method of tumour regression grading (TRG) described by Rubbia-Brandt et al. (2007) is the most widely used for determining histological response to chemotherapy; it categorises tumour response according to the balance of fibrosis and residual tumour. This method has been adapted from response grading of other tumours such as rectal and oesophageal tumours (Mandard et al., 1994). CRLM response to chemotherapy on imaging, meanwhile, is usually performed by CT using RECIST 1.1 criteria (Eisenhauer et al., 2009) although it is recognised that RECIST response criteria may misrepresent morphological tumour treatment response. Reddy et al. (2016) showed a significant proportion of good pathological responders do not have RECIST response, and that pathological response is the more significant predictor of relapse-free survival at multivariate analysis. The prognostic relevance of markers of pathological response and

residual tumour after chemotherapy has been demonstrated in several studies (Maru et al., 2010; Poultsides et al., 2012; Knijn et al., 2013).

Conventional TRG is based on the relationship of fibrosis to residual tumour, considering necrosis to be a phenomenon related to tumour growth and angiogenesis. However, other studies have distinguished this 'dirty' or 'usual' necrosis from treatment-related 'infarct-like' necrosis (Li Chang et al., 2012, Ishida et al., 2015), characterised by large confluent areas of eosinophilic cytoplasmic remnants located centrally within a lesion with absent or minimal admixed nuclear debris. Infarct-like necrosis is found in CRLM following perioperative chemotherapy, in particular bevacizumab, and is thought to be a form of treatment response (Klinger et al., 2010; Wicherts et al., 2011). Although, the potential of MRI or other imaging techniques to actually distinguish different types of necrosis has not been studied, size-based imaging response criteria have been adapted to reflect the fact that tumour necrosis may be a feature of treatment response, particularly in the setting of local therapies or targeted antiangiogenic and immunotherapy agents. For example, the Choi criteria (Choi et al., 2007) were developed for gastrointestinal stromal tumours and have been applied in e.g. RCC (Thian et al., 2014), and mRECIST criteria for hepatocellular carcinoma (HCC) (Lencioni and Llovet, 2010). These incorporate enhancement as well as size criteria, and can reflect response and predict outcome better than conventional response criteria. Similarly, the tissue characterisation of CT or MRI could be used to better assess treatment response in CRLM. This has

predominantly been studied in CRLM treated with the liver-directed therapies transcatheter arterial chemoembolization (TACE) or selective internal radiation therapy (SIRT) (Chapiro et al., 2015; Shady et al., 2016).

Vascular, biliary, lymphatic and perineurial invasion

Vascular invasion is a common feature of CRC and of CRLM. Portal vein invasion occurs in approximately a quarter of cases (reported range 10-49%) and hepatic venous invasion in approximately one tenth of cases (reported range 5-24%) (Knijn et al., 2013). Similarly to primary CRC, venous invasion has been associated with poorer clinical outcomes (Yamamoto et al., 1999; Hayashi et al., 2010). As discussed above, MRI can demonstrate extramural venous invasion of primary rectal cancer. Macrovascular invasion of HCC is common and readily depicted by CT and MRI (Asaoka et al., 2014). Although large vessel invasion is less common in CRLM, adjacent venous invasion can sometimes be observed. Involvement of smaller peritumoral vessels has not been studied for CRLM.

Lymphatic invasion is a less common feature than venous invasion, but has again been shown to be associated with poorer outcome (Shirabe et al., 1997; Sasaki et al., 2002). Lymphatic vessels are smaller than the resolution of current imaging techniques and therefore lymphatic invasion is unlikely to be assessable by MRI, although the presence of periportal, retroperitoneal or more distant lymphadenopathy may be a surrogate marker for this pathological feature. Neither biliary nor perineurial invasion has been associated with adverse clinical

outcomes (Knijn et al., 2013) and there are no imaging studies correlating these features with MRI in CRLM.

Tumour-liver interface and pseudoencapsulation

Similarly to HCC, some metastases have a fibrous pseudocapsule comprised of collagen (Okano et al., 2000). The underlying tumour biology is not well understood. Some authors have reported this to be present in approximately 60% of CRLM (Yamamoto et al., 1999, Okano et al., 2000), although it is not so prevalent in all series (Eefsen et al., 2015), and it has been associated with improved 5-year survival. The presence of a fibrous capsule of CRLM can be visualised by MRI as a ring of low signal intensity on T1 and T2-imaging (Outwater 1991), with late contrast enhancement, although compression and atrophy of peripheral hepatocytes can cause similar appearances. Pre-operative MRI has been shown to accurately distinguish encapsulated HCC from non-encapsulated HCC (Chu et al., 2012), and prospective investigation of the accuracy of MRI in demonstrating pseudoencapsulated CRLM is warranted.

More recently, some authors have further categorised the interface between liver parenchyma and tumour. One classification characterises the interface as 'pushing', 'desmoplastic' and 'replacement' (Vermeulen et al., 2001). Desmoplastic metastases, which include pseudo-encapsulated metastases, have likewise been linked to improved outcomes compared with non-desmoplastic metastases (Siriwardana et al., 2016; Frentzas et al., 2016). Furthermore, the

replacement pattern, which is thought to be related to vascular co-option rather than angiogenic behaviour, has been related to non-response to antiangiogenic agents (Donnem et al., 2013; Siriwardana et al., 2016, Frentzas et al., 2016). It would therefore be of clinical interest, for treatment selection and prognostication, to be able to classify these metastases *in vivo*. As discussed above, although peripheral fibrosis and/or desmoplasia can be demonstrated, the diagnostic performance of MRI for these CRLM subtypes has not been studied.

Functional tumour imaging

There is a recognised framework for understanding the abnormal biological adaptations that characterise tumours (Hanahan and Weinberg, 2011). In the same way that this informs novel therapeutic strategies, it can also provide a framework for novel imaging modalities that may have prognostic and therapeutic implications. Although a diverse range of imaging techniques, particularly molecular imaging, have been used to assess tumour biological processes in the research setting, there are a small number of functional imaging techniques which have been investigated in cancer patients and could be readily incorporated in clinical practice.

Vascular imaging

Induction of angiogenesis is an important feature of metastases. Markers of tumour angiogenesis, such as microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression are associated with poor outcomes, for example in non-small cell lung cancer (NSCLC) (Bremnes, Camps and Sirera, 2006) and

CRC (Guetz et al., 2006). Perfusion imaging techniques, including dynamic contrast-enhanced MRI (DCE-MRI) and perfusion CT (pCT), use multi-phase image acquisition following injection of intravenous contrast agents to measure contrast delivery and uptake into tumours. Modelling derived image data produces several metrics, which relate to tumour vascularity and perfusion (George et al., 2001), although there is heterogeneity of technique and several potential sources of error, particularly for DCE-MRI (Zahra et al., 2007). Attempts to correlate these dynamic imaging data with static histological markers of angiogenesis, such as a MVD, have produced mixed results (Zahra et al., 2007), although response evaluation has been more promising, with several studies reporting correlation of these biomarkers with response to radiotherapy (Surlan-Popovic et al., 2010), chemotherapy (Ah-See et al., 2008) and antiangiogenic therapy (Koukourakis et al., 2007; O'Connor et al., 2012). As yet, despite their potential, these techniques are not used for clinical decision-making in the oligometastatic setting.

Metabolic imaging

Deranged glucose metabolism is recognised as a key adaptation of tumour cells (Warburg, Miles and Williams, 2008). The glucose analogue ^{18}F -FDG is widely used in PET-imaging for detecting metastatic disease, and ^{18}F -FDG-PET/CT is routinely acquired in some patients with suspected oligometastatic cancer. The standardised uptake value (SUV), a semi-quantitative indicator of metabolic activity, is produced for each voxel, and metrics of tumour metabolic activity and

total metabolic tumour burden, the metabolic tumour volume (MTV) and tumour glycolytic volume (TGV), can be derived. High metabolic activity and burden has been found to be a poor prognostic marker in several settings, for example in patients with CRLM undergoing resection, high SUV and TGV are poor prognostic markers (Riedl et al., 2007; Muralidharan et al., 2012). In patients with liver metastases treated with chemotherapy, reduction in FDG-uptake indicates improved prognosis (Xia et al., 2015). As yet there is no threshold to allow decision-making based on these data, but novel methods for analysing PET data may further improve prognostication using this functional imaging tool (Chicklore et al., 2013).

6. *Challenges*

Developing imaging biomarkers for the oligometastatic setting is challenging. Oligometastases may require biomarkers specific to each organ and primary tumour (Zarinabad et al 2014). Differences in the imaging phenotype will occur due to the anatomical location and primary tumour biology, potentially introducing significant variation. As a result, it may prove difficult to validate imaging biomarkers for less common clinical scenarios. Existing biomarkers for oligometastatic cancer have predominantly been derived and confirmed for higher volume and more established surgical interventions.

Compared with blood or tissue-based biomarkers, imaging biomarker studies have often lacked sufficient sample size for validation, particularly for multivariate analysis alongside established clinical biomarkers. Technological advances and differences in equipment and protocols create inconsistencies in imaging within and between institutions, which are then less readily combined as single datasets. Novel imaging, especially molecular imaging, may be expensive or limited, reducing the potential size of datasets.

There are several strategies to address these issues. One is to use semantic features based on conventional imaging, which are less influenced by variable imaging technique, and this has met with some success. However, there are limitations to the data that can be reliably extracted by visual interpretation. With the computing power now available, it is possible to derive numerous quantitative image features, creating mineable, multiparametric data; this is the evolving field of radiomics (Aerts et al., 2014). These feature-sets offer the promise of more consistent analysis, which can be more readily incorporated into clinical and trial workflows, and applied to large retro- and prospective populations for clinical validation. Radiomic data then can also be linked to known histopathological or genomic biomarkers, termed radiogenomics (Aerts et al., 2014). Already, genomic signatures of oligometastatic patients are being described in small cohorts (Lussier et al., 2011; Uppal et al., 2015; Wong et al., 2016; Pitroda et al., 2018). The development and validation of prognostic tools based on high-dimensional imaging data linked to clinical and genomic metadata is promising (e.g. Gevaert

et al., 2012), and there are ongoing efforts to collate imaging biobanks to provide sufficient substrate for validation. Finally, artificial Intelligence (AI) is the rapidly developing field of computer self-learning, and holds great promise for tumour characterization. It requires large validated datasets, as well as further research into its role in cancer imaging, but, in the future, is likely to help characterise oligometastases more consistently than human observers.

Aims and objectives

2.1. Chapter 3

2.1.1. Analyse conventional prognostic imaging biomarkers in a retrospective cohort of patients with resected CRLM.

2.1.2. Explore novel imaging biomarkers derived from ^{18}F -FDG-PET/CT in a retrospective cohort of patients with resected CRLM.

2.2. Chapter 4

2.2.1. Explore the within and between patient variation of morphological features of CRLM

2.2.2. Use immunohistochemical techniques to explore the within and between patient variation of protein expression in CRLM relevant to functional tumour imaging techniques.

2.3. Chapter 5

2.3.1. Perform pCT and DCE-MRI in patients with resectable CRLM, and derive PK parameters.

2.3.2. Compare pCT- and DCE-MRI-derived PK parameters, and semi-quantitative variables derived from ^{18}F -FDG-PET.

2.3.3. Use spatially matched histological specimens to compare functional imaging variables with tumour immunohistochemical staining.

2.3.4. Explore novel subregion analysis of DCE-MRI data and compare this with a histological gold standard.

Chapter 2

Materials and Methods

1. *Functional Imaging of Liver Metastases (FILM) study*

i. Prospective observational trial

The prospective component of the FILM study was conducted at a single tertiary hepatopancreaticobiliary (HPB) centre (Oxford University Hospital NHS Foundation Trust; OUH) between 2012 and 2017.

Patients

15 patients were recruited to this prospective study and provided informed written consent. Consenting adult (>18 years) patients were eligible if they had a diagnosis of CRC, at least one liver metastasis confirmed by imaging criteria or

biopsy, and were planned for surgical hepatic metastasectomy. Patients were ineligible if they had a contraindication to one of the study procedures, or no metastasis measuring at least 8mm in greatest axial dimension.

Although 15 patients were recruited and underwent baseline imaging investigations, one patient (FILM009) was withdrawn due to diagnostic uncertainty about the presence of the suspected liver metastases. The specimens for two patients were not available for analysis. One patient declined post-treatment MRI. A description of the parameters recorded and a summary of the study patients is provided in the tables below.

Table 2a: Summary of demographic and clinical parameters recorded for patients in the prospective and retrospective studies

Parameter	Description
Age	Age at the time of hepatic resection
Sex	Male or female
Primary T-stage	T-stage of the primary tumour based on 8th Edition TNM
Primary N-stage	N-stage of the primary tumour based on 8th Edition TNM
Primary lymph node status	Primary tumour was node positive (N1+) or negative (N0)
Disease free interval	The interval between resection of the primary tumour and the hepatic metastases
Synchronous disease	If the hepatic metastases were considered to be present at the time of diagnosis of the primary tumour

Table 2b: Summary characteristics of prospective FILM study patients (n=14)

Characteristic	Median/Count	
Age	63 years	44-76 years
Sex		
• Female	3	21%
• Male	11	79%
Primary tumour		
• Colon	7	50%
• Rectum	7	50%
Primary T-stage		
• T1	1	7.1%
• T2	1	7.1%
• T3	11	78.6%
• T4	1	7.1%
Primary lymph node status		
• Positive	6	42.9%
• Negative	8	57.1%
DFI from primary tumour diagnosis	457 days	0-1129 days
Synchronous disease	2	14.2%

Study procedures

All patients were discussed at the Liver Multidisciplinary Team (MDT) meeting and the decision was made to offer hepatic metastasectomy. All patients had routine standard of care staging investigations comprising CT of the chest, abdomen and pelvis (CAP), liver MRI and ¹⁸F-FDG-PET/CT. Patients consenting for the trial additionally had DCE-MRI and pCT of the liver, typically timed to coincide with another routine clinical visit. Some patients received four cycles of neoadjuvant chemotherapy over three months prior to surgical resection. For these patients it is routine clinical practice to repeat the CT of the chest, abdomen and pelvis. Study patients additionally underwent repeat DCE-MRI and pCT of the liver at the

same time. The methodology for pCT and DCE-MRI of the liver is discussed below.

It was the intention for all patients who entered the study to proceed to surgical metastasectomy. The tissue was collected and sectioned to allow direct imaging-histopathology correlation as discussed below.

Table 2c: Summary of the prospective FILM study patients (n=14)

Patient	# metastases	Involved liver segments	Neoadjuvant chemotherapy	Hepatic resection
FILM001	2	6 ¹ and 7	Yes	Right hemihepatectomy
FILM002	1	4	No	Extended left hemihepatectomy
FILM003	2	8 ¹ and 6	No	Non-anatomical
FILM004	1	2	No	Left lobectomy
FILM005	1	7	No	Non-anatomical
FILM006	1	5	No	Non-anatomical
FILM007	1	4	Yes	<i>Extended right hemihepatectomy</i> ²
FILM008	2	6 ¹ and 6	No	Non-anatomical
FILM010	2	4 ¹ and 4	Yes	Non-anatomical
FILM011	1	4	No	Non-anatomical
FILM012	1	5	Yes	<i>Non-anatomical</i> ²
FILM013	1	5	No	Non-anatomical
FILM014	1	7	No	<i>Right hemihepatectomy</i> ²
FILM015	1	7	No	Non-anatomical

Notes

1. Where two metastases present, the lesion in bold was the target of functional imaging
2. Where hepatic resection italics, the specimen was not available for analysis

ii. Retrospective cohort study

The retrospective component of the FILM study was based on a population of patients who had undergone surgical liver metastasectomy for CRLM at a single HPB tertiary referral centre between 2010 and 2014.

Patients

There were 87 patients (table in this cohort which was combined with nine patients in the prospective study to produce a cohort of 96 patients (Table 2d and Appendix 2). Patient demographics and details of the primary tumour and secondary recurrence were extracted as detailed above.

Conventional retrospective imaging and tissue

For these patients the routine staging investigations includes CT CAP, MRI liver and ^{18}F -FDG-PET/CT. 58 (60.4%) patients went on to have neoadjuvant chemotherapy. These patients typically had repeat CT CAP, but may have additionally had repeat liver MRI and ^{18}F -FDG-PET/CT depending on clinical requirements. The available imaging is summarised in Appendix 1.

Table 2d: Summary characteristics of patients in the retrospective FILM study (n= 96)

Characteristic	Number (unless stated)	Percentage (unless stated)
Median age (range)	63 years	(22-85) years
Primary tumour		
• Colon	53	55.2%
• Rectum	43	44.8%
Primary tumour stage		
• T1	2	2.1%
• T2	5	5.2%
• T3	48	50.0%
• T4	22	22.9%
• Unknown	19	19.8%
Primary tumour lymph node status		
• Positive	59	61.5%
• Negative	19	19.8%
• Unknown	18	18.8%
Disease free interval (average (median) and range)	264 (21) days	0-2385 days
Synchronous disease	52	54.2%
Number of metastases (average (mode) and range)	2.45 (1)	1-10
Neoadjuvant chemotherapy	58	60.4%

Tissue samples

Some patients in the retrospective cohort had provided consent for tissue biobanking. For these patients (n = 40), sections of all archived metastasectomy tumour specimens were cut. Histopathological processing and assessment is discussed below. The available histopathological tissue is summarised in Appendix 1.

2. *Imaging acquisition*

i. Routine CT and perfusion CT

Routine CT CAP is performed after intravenous injection of iodinated contrast medium followed by a saline flush with imaging performed as a volumetric spiral acquisition 60-70 s after the start of the injection. For patients undergoing pCT at the same time as CT CAP, the CT CAP was performed immediately after the pCT, using the same contrast bolus. Some staging investigations were carried out outside the study institute and were therefore performed using variable equipment, scanning parameters and technique.

Detailed scanner settings for the pCT are included in Appendix 3. All pCT studies were performed on a 64-slice GE CT (LightSpeed VCT, GE Healthcare, Amersham, UK). For pCT, an unenhanced volumetric CT of the upper abdomen was performed in end inspiration through the upper abdomen to incorporate the entire liver, to allow planning of the post-contrast acquisition. The maximal craniocaudal coverage of the pCT study is 4cm; the planned acquisition was centred on the midpoint of the tumour. The pCT acquisition was performed following intravenous injection of 70mL iodinated contrast medium (Omnipaque 300, GE Healthcare, Amersham, UK) followed by a 20mL saline flush via a pump injector (Medrad Stellant Dual Syringe CT Injection System, Warrendale, PA, USA) at a rate of 5 ml/s. pCT was performed for 45 seconds, beginning 10 seconds after the start of the contrast medium injection using the following parameters:

eight contiguous sections, collimated to 5 mm, were obtained at 1-second intervals through the tumour using cine-mode acquisition (120 kV, 60 mA). Patients were scanned during a single breath hold in end inspiration after coaching from the radiography staff.

ii. Routine liver MRI and DCE-MRI

Routine liver MRI is a multiparametric MRI incorporating axial and coronal T2 weighted (T2W) imaging, T1 weighted (T1W) imaging including, opposed phase imaging, diffusion weighted (DWI) and multiphase T1W imaging after injection of gadolinium chelate contrast medium, typically incorporating arterial, venous and delayed phase imaging. Some staging investigations were carried out outside the study institute and were therefore performed using variable equipment, scanning parameters and technique.

Detailed scanner settings for the DCE-MRI protocol are included in Appendix 4. In brief, DCE-MRI studies were performed at 1.5T (GE HDX Twinspeed MR scanner; GE, Milwaukee, WI) with an 8-channel body coil. Following some routine sequences including axial and coronal T2W imaging, T1W imaging, and DWI, DCE-MRI was performed. All DCE-MRI imaging was performed in the axial plane. Multiple flip-angle (15°, 9° and 3°) sequences to calculate voxel $T1_0$ values followed by T1W imaging (axial 3D multiphase LAVA; field of view = 42, slice thickness = 5.0 mm, location per slab = 22, overlap = 0, TR = 4.46 s, TE = 2.128 s, Cartesian k-space trajectory) of a predefined volume centered on the largest

liver metastasis before and after the injection of a 15 mL bolus of gadolinium chelate (Gadoteridol; ProHance, Bracco Diagnostics, Princeton, USA), with a 15 mL saline flush, at 3 mL/s, which was started at the midpoint of the third acquisition. All studies had at least 20 acquisitions. The timings of the DCE-MRI acquisitions are shown in full in Appendix 5. Volumes were acquired over a 7s breath-hold during end-expiration for at least 7 minutes after contrast administration, with lengthening interscan intervals (0-2 minutes – 1 breath cycle between each acquisition; 2-4 minutes: 2 breath cycles between each acquisition; 4-6 minutes: 3 breath cycles between each acquisition; 6+ minutes: 4 breath cycles between each acquisition). The number of acquisitions was therefore principally determined by respiratory rate.

iii. ^{18}F -FDG-PET/CT

^{18}F -FDG-PET/CT scans were performed on a 3D mode time of flight (ToF) GE Discovery 690 PET/CT system (GE Healthcare, Milwaukee, USA). The patients were fasted for at least 6 h prior to their scan. Their blood glucose was measured prior to intravenous injection with 4 MBq/Kg ^{18}F -FDG. Imaging commenced 90 min post-injection and covered a minimum of skull base to upper thigh. The ^{18}F -FDG-PET/CT images were acquired under normal tidal respiration for four minutes per bed position. The standard PET reconstruction algorithm used is ToF ordered subset expectation maximisation (OSEM) with two iterations, 24 subsets, and a 6.4mm Gaussian filter (VPFX, GE Healthcare, Milwaukee, USA). The ^{18}F -

FDG-PET data is attenuation-corrected using the contemporaneously-acquired unenhanced CT.

3. *Imaging analysis*

i. Conventional imaging data

CT CAP assessment

The presence of any extrahepatic metastatic disease was based on the clinical CT and ^{18}F -FDG-PET/CT reports.

Liver MRI assessment

The routine liver MRI was used to determine the number, size and location of the hepatic metastases. The size recorded was the largest dimension of the largest metastasis before any treatment. Unilobar disease was defined as disease confined to Couinaud segments 2-4 (left lobe disease) or segments 5-8 (right lobe disease). Bilobar disease was defined as disease within segments 2-4 and segments 5-8.

For patients where the histological specimens were available for comparison the MRI studies were also assessed for the presence of pre-specified morphological features.

¹⁸F-FDG-PET analysis

The presence of any extrahepatic metastatic disease was based on the clinical CT and ¹⁸F-FDG-PET/CT reports.

The reconstructed attenuation corrected ¹⁸F-FDG-PET data and corresponding CT were loaded in a dedicated nuclear medicine workstation (HERMES; Nuclear Diagnostics, Stockholm, Sweden). For each ¹⁸F-FDG-PET/CT study semi-quantitative measurements were recorded. The PET data is presented as a spatial map of voxels which are attributed standardised uptake values (SUV); semi-quantitative representations of FDG-avidity. SUVmax is the highest voxel value in a region of volume of interest (ROI/VOI), SUVmean is the mean value of all voxels within a ROI/VOI and SUVpeak is the highest mean value of a 1 cm³ subregion of a ROI/VOI, generated automatically by the HERMES software. Using methodology described in our prior work (Parvizi et al., 2015), a three-centimetre diameter spherical VOI was placed within a normal background liver parenchyma unaffected by liver metastatic disease, typically in the right lobe of the liver, avoiding large photogenic vascular structures. This was used to derive the background liver SUVmax, SUVmean and the standard deviation of SUV of the background liver parenchyma (background SUVdeviation). Using a variable threshold for each PET study to define tumour, calculated as 110% of the background liver SUVmax for that study, a VOI for every metastasis was segmented automatically. Using a threshold just above background SUVmax was designed to exclude FDG-activity in normal liver parenchyma from the tumour

VOIs. Where tumour abutted normal normal structures which are FDG-avid, such as the myocardium, these were manually excluded from the tumour VOI based on the CT imaging. For each metastasis, SUVmax, SUVmean and SUVpeak were derived from the segmented volume (Wahl et al., 2009). The total volume of each metastasis is reported by the software, the 'metabolic tumour volume' (MTV). All tumour VOIs within the liver were merged and the total MTV (MTVtotal) generated for each patient. The SUVmax, SUVmean and SUVpeak for the merged VOIs were recorded. The 'tumour glycolytic volume' (TGV) was calculated for each patient as the product of the MTVtotal and SUVmean for the merged VOIs. A summary of the extracted parameters is provided below (Table 2e).

Table 2e: Summary of extracted 18F-FDG-PET parameters

¹⁸F-FDG-PET parameter	Description
Background SUVmax	Maximum SUV within the VOI placed in the background liver
Background noise	Standard deviation of SUV values within the VOI placed in the background liver
Threshold	Calculated as $1.1 * \text{Background SUVmax}$
SUVmax	Maximum pixel SUV value within each tumour VOI
SUVpeak	Average pixel SUV value for highest 1cm ³ subregion of each tumour VOI
SUVmean	Average pixel SUV value for each VOI
MTV	The volume of each VOI (cm ³)
TGV	The product of MTV and SUVmean (cm ³)

Response assessment

For patients who underwent neoadjuvant chemotherapy the response was assessed by comparing pre- and post-treatment imaging. Where a pre- and post-treatment liver MRI was available, these were compared. Where post-treatment CT was available only, CT was used for this assessment. Response assessment was based on RECIST 1.1 criteria for hepatic response (Eisenhauer et al., 2009). Heterogeneous response was defined as where the response of individual liver metastases fell into different RECIST response categories. Insufficient numbers of patients had pre- and post-treatment ^{18}F -FDG-PET/CT to allow analysis of PET-response.

ii. Functional imaging analysis

Perfusion CT

DICOM data from the pCT acquisition was analysed using commercially-available software (Perfusion 4.0; GE Healthcare Technologies) using the Liver Tumour Protocol which uses an algorithm based on a dual vascular input function to reflect the dual blood supply to the liver. All 5-mm axial images were chosen, and a processing threshold of -20 to 105 HU was selected. The arterial input function (AIF) was derived by manually defining a 12-25mm³ ROI in the abdominal aorta and the portal venous input function (PVIF) was derived by manually defining a 12-25mm³ ROI in the main portal vein or one of its branches. The arterial and portal venous time-enhancement curves for the acquisition were automatically generated and the final image before arrival of the arterial contrast bolus defined.

The software analysed the entire pCT volume (eight 5mm axial sections) and produced pharmacokinetic parameter maps of blood flow (BF), blood volume (BV), mean transit time (MTT), peak enhancement (PE), time to peak enhancement (TTP) and hepatic arterial fraction (HAF).

Table 2f: Summary of extracted pCT parameters

pCT parameter	Description
BF	Blood flow (ml/100g.min)
BV	Blood volume (ml/100 mg)
MTT	Mean transit time (s)
PE	Peak enhancement (Hu)
TTP	Time to peak enhancement (s)
HAF	Hepatic arterial fraction (%)

The pCT parameter maps were exported as DICOM images and imported into dedicated DICOM viewing software (Osirix, Pixmeo SARL, Bernex Switzerland). Regions of interest were drawn on sequential axial sections to extract the pCT parameters from the entire tumour volume for all parameter maps. These were recorded, along with the voxel count at each axial section, and weighted means calculated to produce a final value based for each pCT parameter for the entire tumour volume. The parameter values for the central slice for each metastasis were also recorded for correlation with the DCE-MRI and IHC data.

DCE-MRI

a. Motion correction

The liver is subject to respiratory motion. Although images were acquired in end expiration, which minimizes inter-frame motion (Kimura et al., 2004), there was residual inter-frame motion due to breath-hold variability. This was corrected using a non-rigid motion correction algorithm developed alongside this project, using a subset of the acquired liver DCE-MRI studies ($n=4$), which results in significant improvement in the registration error (Papiez et al., 2015). Briefly, markers were placed on consistent identifiable landmarks within the liver contour (focal lesions and vascular bifurcations) and on the liver surface at each of the 20-30 acquisitions during the DCE-MRI study for four patients in the prospective FILM study. The first pre-contrast image was the reference image. These were used to describe liver motion and to develop the motion correction algorithm which was then applied to the entire dataset. The initial target registration error (TRE), the average distance from baseline of each of the registration points in subsequent acquisitions, was 7.83mm which was reduced to 3.28mm following registration.

b. Pharmacokinetic analysis

The variable flip angle sequence was registered to the baseline image of the DCE-MRI image and used to derive baseline T1 values ($T1_0$) and, for each voxel in the DCE-MRI acquisition, signal was converted to contrast concentration. The Tofts' model (Tofts et al., 1999), using population-average PVIF and AIF (Orton et

al., 2009), was fitted to the contrast enhancement curve of each voxel to produce maps of PK parameters.

c. Extraction of PK parameters

The PK parameter maps were imported into proprietary in-house PK View software, which was developed to aid this analysis (Irving et al., 2016). Mean, median, deviation, minimum and maximum values for K^{trans} , k_{ep} , and $T1_0$ for each of the defined ROIs and VOIs were derived.

Table 2g: Summary of extracted dceMRI parameters

DCE-MRI parameter	Description
K^{trans}	The volume transfer constant reflecting the rate of efflux of contrast from the plasma into the EES (min^{-1})
k_{ep}	The volume transfer constant reflecting the rate of reflux of contrast from the EES into the plasma (min^{-1})
$T1_0$	The baseline tissue T1 value before contrast enhancement (ms)

4. Histopathological Analysis

i. Tissue processing

Patients in the prospective FILM study

For patients in the prospective FILM study attempts were made to ensure the histological cut-up matched the functional imaging; required sectioning in the axial plane. At the the time of hepatic resection the specimen was oriented perpendicular to the craniocaudal axis by the operating surgeon and the anterior

surface marked. The midpoint of the metastasis in the craniocaudal axis was identified using ex-vivo ultrasound and marked superficially on the specimen. For deep lesions, an ultrasound examination was performed at the time of histological cut-up.

Histopathological cut-up was performed prior to formalin fixation. Liver specimens were sectioned in the axial plane at the meridian of the selected metastasis based on the surgical and sonographic landmarks, with further approximately five millimeter sections taken through the metastasis. The sections were marked (anterior, posterior, medial and lateral), photographed, and then transferred to cassettes. Sections were fixed overnight in formalin and embedded in paraffin. Three micrometer sections of each metastasis at the midpoint section were cut and stained using haematoxylin and eosin (H&E), and mounted on glass slides. Further contiguous unstained sections were cut from each metastasis for IHC staining.

For two patients (FILM001 and FILM010) the cut-up was not supervised and the specimen was processed. Metastasectomy tissue was not collected for two patients FILM012 and FILM014.

Other liver resection specimens

These were processed as per the normal Oxford University Hospitals Cellular Pathology laboratory, which involves overnight fixation in formalin followed by

careful section at five millimeter intervals through the resection specimen with representative sections of tumour(s) and normal liver taken and transferred to cassettes and embedded in paraffin. Three micrometer sections of each metastasis at the midpoint section were cut and stained using haematoxylin and eosin (H&E), and mounted on glass slides. Further contiguous unstained sections were cut from each metastasis for IHC staining.

ii. Morphological features

H&E stained slides were reviewed, using light microscopy, jointly by two consultant histopathologists with >10 years experience in reporting CRC histopathology. Each metastasis was scored by consensus, blinded to all clinical and imaging data, for series of pre-defined features using a specifically-designed proforma (see Appendix 6).

iii. Immunohistochemical (IHC) staining and analysis

Except for CD34 staining, all antibody staining was performed on a Leica BOND-MAX Autostainer (Leica Microsystems GmbH, Wetzlar, Germany). Glass-mounted FFPE sections were baked at 72 °C, dewaxed, washed and pre-treated with an Epitope Retrieval Solution (ERS), either ERS1, a citrate-based solution at pH 6.0, or ERS2, a EDTA-based at pH 9.0) at 98 °C for 10 or 20 minutes to facilitate heat-induced epitope retrieval (HIER). After washing with alcohol and wash buffer, a peroxidase block was carried out for 10 min using the Bond Polymer Refine

Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were again washed and then incubated with the primary antibody for 30 min. Subsequently, tissues were incubated with polymer for 10 min and developed with DAB-Chromogen for 10 min.

GLUT1 staining optimisation and protocol

GLUT1 staining was performed using the SPM498 mouse monoclonal anti-human (Abcam, Cambridge, UK). This antibody had not previously been optimised on the Leica BOND-MAX. Therefore, a series of optimisation steps were performed using control and test tissue and the staining reviewed after each run.

Run 1: Dilution 1:100, 1:200 and 1:400. HIER performed using ERS1 and ERS2 at 98°C for 20 minutes followed by incubation with the primary antibody for 30 minutes. Tonsil control tissue was staining. Good staining throughout but significant background staining even at 1:400 dilution.

Run 2: Dilution 1:1000 and 1:2000. HIER performed using ERS2 at 98°C for 10 and 20 minutes followed by incubation with the primary antibody for 30 minutes. Paired tonsil control tissue and liver and rectum test tissues were stained. 1:1000 dilution with 10 minute incubation preferred in rectum but some cytoplasmic staining observed at 1:1000 in liver.

Run 3: Concentration 1:1000 and 1:2000. HIER performed using ERS2 at 98°C for 10 minutes followed by incubation with the primary antibody for 30 minutes. Four paired liver test tissue samples were stained.

The final run was reviewed by a Consultant Histopathologist. For experimental tissue the GLUT1 antibody was used at a concentration of 1:1000. HIER performed using ERS2 at 98 °C for 10 minutes followed by incubation with the primary antibody for 30 minutes.

ki67 staining protocol

Ki67 staining was performed using the MIB-1 mouse monoclonal anti-human antibody (DAKO, Glostrup, Denmark) antibody was used at a concentration of 1:200. HIER performed using ERS2 at 98 °C for 20 minutes followed by incubation with the primary antibody for 30 minutes.

Carbonic anhydrase IX (CA9) staining protocol

The M75 antibody (gift from Professor Adrian Harris Lab) was used at a concentration of 1:200. HIER performed using ERS2 at 98 °C for 20 minutes followed by incubation with the primary antibody for 30 minutes.

Analysis of GLUT1, ki67 and CA9 IHC

Stained slides were scored by two experienced Consultant Histopathologists in consensus for the percentage immunoreactive cells in a hotspot region viewed at

x400 magnification. The strength of staining (strong vs weak) was also assessed. The median for the population was calculated. Metastasis with scores greater than the median were considered 'high' and those lower than the median considered 'low' (Sirwardana et al., 2016).

CD34 staining protocol

Slides were stained for CD34 by the Oxford Centre for Histological Research (OCHRe) using their standard laboratory technique. The mouse monoclonal anti-human QBEnd 10 antibody (DAKO, Glostrup, Denmark) was used at a concentration of 1:200. HIER performed using ERS2 at 98°C for 20 minutes followed by incubation with the primary antibody for 30 minutes.

Analysis of microvessel density (MVD)

MVD was scored using the Chalkley method as previously described (Chalkley, 1943, Badoux et al., 2008). The three most vascular hotspots of tumour, based on screening at x40 and x100 magnification, were selected and viewed at x200 magnification. A 25-point Chalkley eyepiece graticule (Leitz Orthoplan; Leica) was applied to each hotspot with giving a grid area of 0.1886mm². The graticule was orientated to hit the maximum number of tumour microvessels and the number recorded as the Chalkley count. This was repeated for three hotspots and the mean taken.

Chapter 3

Prognostic imaging biomarkers in oligometastatic CRLM

1. Introduction

As described in Chapter 1, radiological imaging techniques are an integral part of the normal clinical workup of patients with oligometastatic CRC. Imaging is invaluable in planning targeted liver-directed therapy, demonstrating the sites, size and anatomical relationships of intrahepatic metastases. Imaging also contributes prognostic information, which helps in selecting patients appropriately for liver-directed therapy. Clinical and radiological prognostic factors have been combined in several prognostic scoring systems (Spelt et al.,

2012), and have been subjected to meta-analysis (Kanas et al., 2012, Numerous studies have demonstrated that a higher number of CRLM confers an adverse prognosis (Spelt et al., 2012, Kanas et al., 2012,), although one study suggested this was related to challenges in achieving clear surgical resection margins with higher numbers of metastases (Scheele et al., 1995, Rees et al., 2008), rather than, as is more commonly hypothesised, due to the number of metastatic events indicating adverse tumour biology. The size of metastases at baseline is variably found to be prognostically relevant, but at meta-analysis larger metastases are associated with poorer outcome (Kanas et al., 2012). The presence of extrahepatic disease, for example lung or peritoneal metastases, is generally associated with worse outcomes (Kanas et al., 2012), although can sometimes be treated successfully with staged or synchronous procedures. Despite adverse prognosis relative to liver-only disease, some patients with extra hepatic metastases from CRC have good long-term outcomes after staged or synchronous resection or ablation (Adam et al., 2009, Hwang et al., 2014, Hadden et al., 2016). Finally, some patients with CRLM receive neoadjuvant chemotherapy, based on evidence of improved outcomes after FOLFOX chemotherapy (Nordlinger et al., 2013), although the evidence base is limited (Khoo et al., 2016), and practice varies across centres. Often, the decision to treat with neoadjuvant chemotherapy is based on the presence of adverse prognostic features, where it can be used as a temporising measure to assess disease behaviour over 4-8 cycles of chemotherapy. Disease progression, or the failure of disease control following neoadjuvant chemotherapy is a significant adverse prognostic feature, and is

generally considered to be a contraindication to radical treatment (Adam et al., 2004). Conversely, response to chemotherapy is a good prognostic indicator (Chua et al., 2010) although it is recognised that conventional imaging response assessments, such as RECIST 1.1 (Eisenhauer et al., 2009), which are based on size criteria, may underestimate response CRLM, particularly to novel biological agents (Shindoh et al., 2013).

There have been various attempts to develop prognostic models based on these individual prognostic factors. For example, Rees et al. (2008) developed the “Basingstoke Predictive Index” based on seven independent prognostic factors in their analysis of 1005 hepatic metastasectomies in 929 patients: the primary tumour lymph node status, primary tumour differentiation, CEA level at hepatectomy, number of hepatic metastases, size of the largest metastasis, the presence of extrahepatic metastatic disease and, post-operatively, whether the resection margin was positive or negative. The highest risk pre-operative groups only had a 2% 5-year cancer-specific survival, compared with 64% for the lowest risk group. However, subsequent studies have not found that this or other models perform adequately in the setting of modern diagnostic and therapeutic approaches, and do not allow patients to be stratified for treatment (Roberts et al., 2014).

Developing improved prognostic biomarkers would be helpful for selecting patients for the growing number of therapeutic options available to clinicians treating patients with oligometastatic cancer.

As set out in Chapter 1, ^{18}F -FDG-PET/CT has been explored as a prognostic biomarker in a number of tumour settings, including oligometastatic CRLM (Xia et al 2015). In this Chapter existing prognostic imaging biomarkers and novel biomarkers derived from ^{18}F -FDG-PET are assessed in a cohort of patients with resected CRLM.

2. Chapter Methods

i. Patient cohort

As described in Chapter 2, these patients were drawn from a retrospective cohort of patients with CRLM who underwent surgical resection at a tertiary HPB centre. 96 patients were included in the analysis with a median follow-up duration of 730 days.

Table 3a: Summary characteristics of patients in the retrospective FILM study

Characteristic	Number (unless stated)	Percentage (unless stated)
Median age (range)	63 years	(22-85) years
Primary tumour		
• Colon	53	55.2%
• Rectum	43	44.8%
Primary tumour stage		
• T1	2	2.1%
• T2	5	5.2%
• T3	48	50.0%
• T4	22	22.9%
• Unknown	19	19.8%
Primary tumour lymph node status		
• Positive	59	61.5%
• Negative	19	19.8%
• Unknown	18	18.8%
Disease free interval (average (median) and range)	264 (21) days	0-2385 days
Synchronous disease	52	54.2%
Number of metastases (average (mode) and range)	2.45 (1)	1-10
Neoadjuvant chemotherapy	58	60.4%

ii. Patient demographics and clinicopathological factors

Patient demographics, clinical parameters and relevant features of the primary tumour and secondary recurrence, are shown in Table 3a and in detail in Appendix 2.

iii. Clinical outcomes

Follow-up was performed through the patients' electronic health record. Patients were censored at the time of last follow-up or at the time of recurrence, whichever was first. The following clinical outcome measures were defined:

- *Hepatic progression-free survival (hPFS):*
 - Baseline: date of incident hepatic metastasectomy
 - Censor: date of first recurrence in the liver
- *Disease-free survival (DFS):*
 - Baseline: date of incident hepatic metastasectomy
 - Censor: any disease recurrence.

Patients with extrahepatic disease present at the time of metastasectomy which was planned for surgical resection but did not undergo planned staged therapy, and were therefore never rendered disease-free, were excluded from the disease-free survival analysis, but included in the hPFS analysis. Patients who underwent planned staged treatment of multisite disease were considered to have been

rendered disease-free, and recurrence was calculated from the time of incident hepatic metastasectomy.

iv. Image analysis

The routine imaging was assessed for the size, number and location of metastases, and any response to treatment as described in Chapter 2. The presence of EHD was recorded based on pre-operative CT and ^{18}F -FDG-PET/CT. For each pre- and, where relevant, post-chemotherapy ^{18}F -FDG-PET/CT study a panel of ^{18}F -FDG-PET parameters was derived as described in Chapter 2.

v. Statistical analysis

Analysis was performed with statistical support from Dr Christiana Kartsonaki from the Clinical Trials and Services Unit and Epidemiological Studies Unit (Nuffield Department of Population Health). Analysis was performed in R (R Foundation for Statistical Computing, Vienna, Austria, 2013). A Cox proportional hazards model was used to perform a univariate analysis of each of the considered clinical and imaging variables for DFS and hPFS, using $p < 0.05$ to determine significance. All continuous imaging variables are standardised to have unit standard deviation such that any estimates are per one standard deviation increase. Correlations of variables were assessed and highly correlated variables were replaced with the first variable and a composite variable of their difference. Multivariate modelling was performed using a regularised Cox regression model initially using all

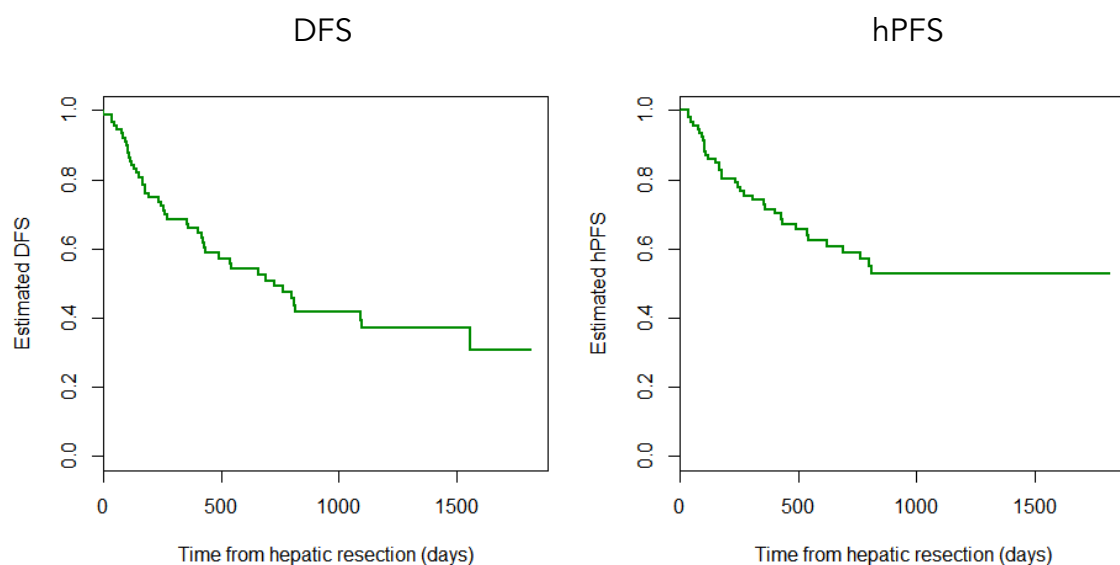
variables, attempting to reach a model in which all variables were significant or considered likely to have an important effect. Secondly, starting from a model which includes all variables that were significantly ($p < 0.1$) associated with DFS in the univariate models, variables are removed one at a time until a subset of variables significantly associated with the outcome were identified.

3. Results

i. Recurrence

96 patients were included in this study, with a median follow-up of 730 days (range 0 - 1838 days). The characteristics of the patients is presented in Table 2a. There were 51 recurrences during the follow-up period with a median time to recurrence of 191 days (range 0 - 1560 days). There were 37 intrahepatic recurrences. Median hPFS was 207 days (range 34 - 811 days). The overall Kaplan-Meier curves for DFS and hPFS are shown in Figure 3a.

Figure 3a: Kaplan-Meier curves for DFS and hPFS for the entire cohort



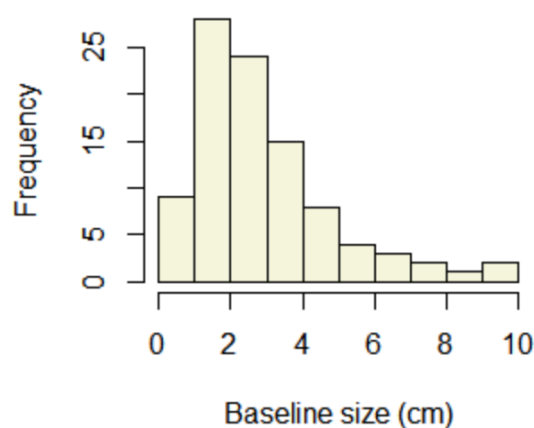
ii. Conventional imaging biomarkers

The imaging biomarkers are summarised in Table 3a. The size of the largest metastasis varied from 0.5 to 10 cm. A histogram of the size of the metastases is shown below in Figure 3b. There was a skew towards smaller metastases in this population, likely a result of clinical selection for treatment for metastasectomy.

Table 3b: Summary of conventional imaging biomarkers in the retrospective FILM study

Characteristic	Mean (unless stated)	Standard Deviation (unless stated)
Size of largest metastasis (cm)	3.06	1.98
Bilobar metastases	19	19.8%
Number of metastases	2.43	2.06
• Median number of metastases	2	
• Patients with >1 metastasis	52	54.2%
• Patients with >3 metastases	22	22.9%
Patients with EHD	8	8.33%

Figure 3b: Histogram of sizes of largest metastases



The modal number of metastases was one (45.8%), although 22.9% of patients had four metastases or more. 19.8% of patients had bilobar disease, although it is not possible for patients with single metastases to have bilobar disease. 36.5% of patients with two or more metastases had bilobar disease. A skew towards unilobar disease, even when multiple metastases were present, may simply be a reflection of liver volumetry, since the right lobe is approximately 2/3rds of the

liver volume (Leeluudomlipi et al., 2002). A minority of patients had EHD at the time of hepatic resection.

iii. ^{18}F -FDG-PET biomarkers

The ^{18}F -FDG-PET biomarkers are summarised in Table 3b. No baseline PET data were available for 8 patients. There was no FDG-avid tumour in 2 (2.1%) patients and these were excluded from the analysis of the tumour PET variables, although background variables were derived. For 4 (4.2%) patients the raw imaging data was not available for analysis. SUVmax could be derived from the clinical report, but none of the other PET parameters could be derived. The number of missing values of SUVpeak was higher than the other variables because it requires a 1cm^3 volume of tumour.

Table 3c: Summary of FDG-PET parameters

Characteristic	Mean (unless stated)	Standard Deviation (unless stated)
Tumour PET features		
• SUVmax	10.32	4.55
• SUVpeak	9.16	3.55
• SUVmean	5.15	1.22
• MTV (mL)	46.58	88.63
• TGVmax (mL)	708.54	1881.43
• TGVmean (mL)	277.75	567.75
Background liver PET features		
• SUVmax	2.93	0.49
• Standard deviation SUV	0.22	0.05

Notes:

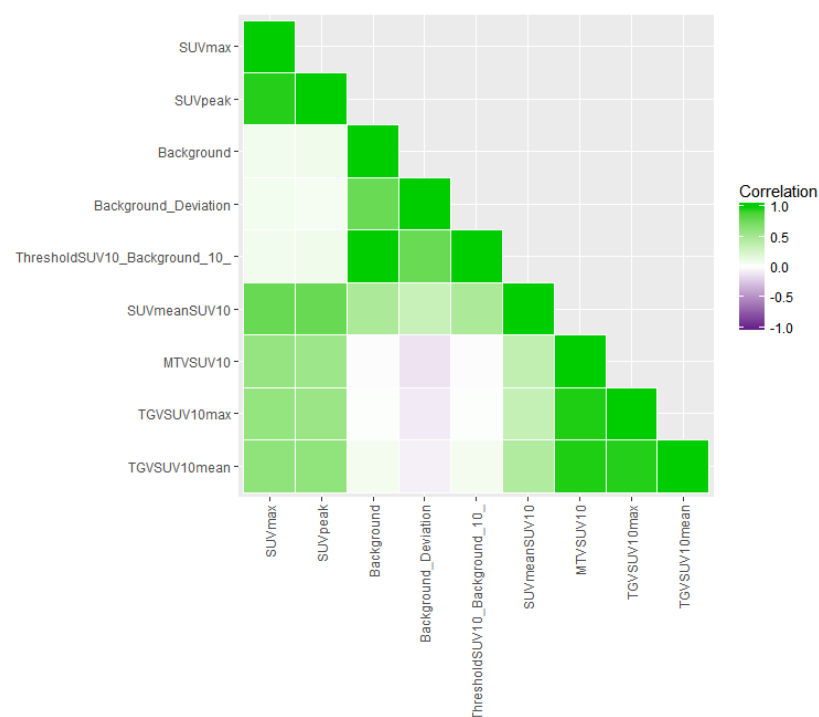
SUV = standardised uptake value in VOI

MTV = metabolic tumour volume

See Chapter 2 for definitions of PET parameters

Correlations of the ^{18}F -FDG-PET derived parameters are shown in Figure 3c below. Several of the ^{18}F -FDG-PET parameters were highly correlated, specifically SUVmax and SUVpeak (0.97), and the two methods for calculating TGV, TGVmean and TGVmax (0.97). In the multivariate analysis, one of each of these pairs was dropped, and replaced with the subtraction of one variable for another.

Figure 3c: Correlation matrix of FDG-PET imaging variables



iv. Univariate analysis

Disease-free survival

The outcomes of the univariate analysis of variables for DFS is shown in Table 3d. Several of the clinical variables were significantly or borderline significantly associated with poorer outcomes, including a node positive primary (HR = 2.56,

$p = 0.033$), T-stage (HR = 1.69 for each increase in T-stage above T1, $p = 0.061$) and a shorter disease free interval (HR = 0.73 for each disease free year, $p = 0.061$). Conventional imaging variables were also significantly associated with poorer DFS, including higher number of metastases (HR = 1.15 for each additional metastasis, $p = 0.016$), bilobar disease (HR = 2.09, $p = 0.035$), and the presence of EHD (HR = 2.98, $p = 0.040$). Kaplan-Meier curves for these six variables are shown below (Figure 3d).

Hepatic progression-free survival

The outcomes of the univariate analysis of variables for hPFS is shown in Table 3d. Again, a node positive primary (HR = 3.62, $p = 0.034$) and a shorter disease free interval (HR = 0.59 for each disease free year, $p = 0.034$) were significantly associated with poorer hPFS. There was no significant effect of T-stage on hPFS. For hPFS the greater number of metastases (HR = 1.25 for each additional metastasis, $p < 0.0001$) and bilobar disease (HR = 3.80, $p < 0.0001$) had an effect on outcome. There was no significant effect of EHD on hPFS. There was a borderline significant effect of SUVmean on hPFS (HR = 1.41 per standard deviation from mean, $p = 0.068$). Kaplan-Meier curves for these five variables are shown below. The curves for SUVmean split by median did not separate and so curves split by tertiles were also created. The outcomes in first and third tertile were more closely related, suggesting a bimodal effect of the variable on outcome. Similar patterns were seen for SUVpeak, MTV and TGVmean and TGV max. In a post-hoc analysis, based on review of the Kaplan-Meier curves, a

comparison of Background SUVmax above and below the median was performed, which was borderline significant (HR = 2.04, $p = 0.069$).

Figure 3d: Kaplan-Meier curves of DFS for patients with resected CRLM grouped by selected clinical and radiological variables

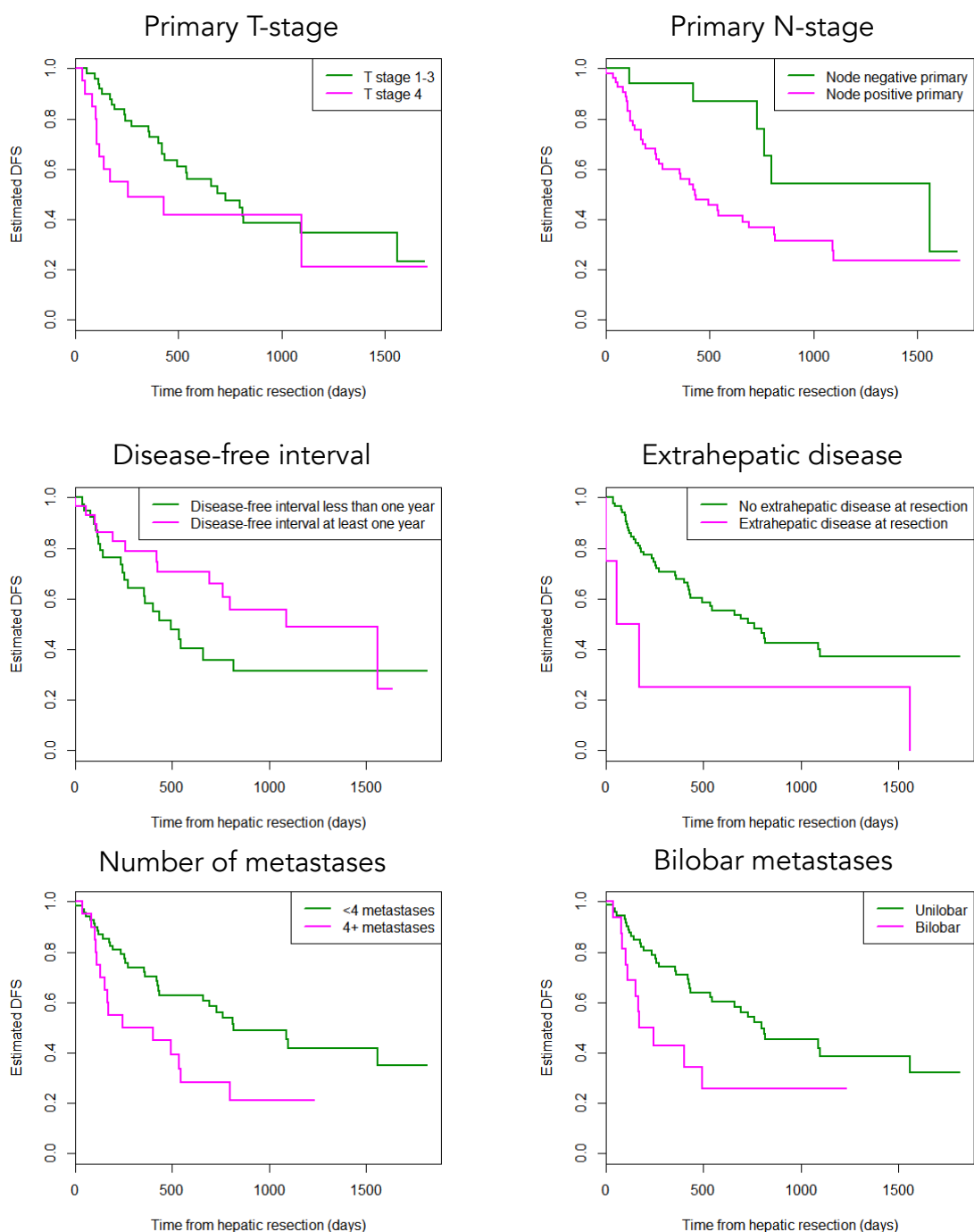


Figure 3e: Kaplan-Meier curves of hPFS for patients with resected CRLM grouped by selected clinical and radiological variables

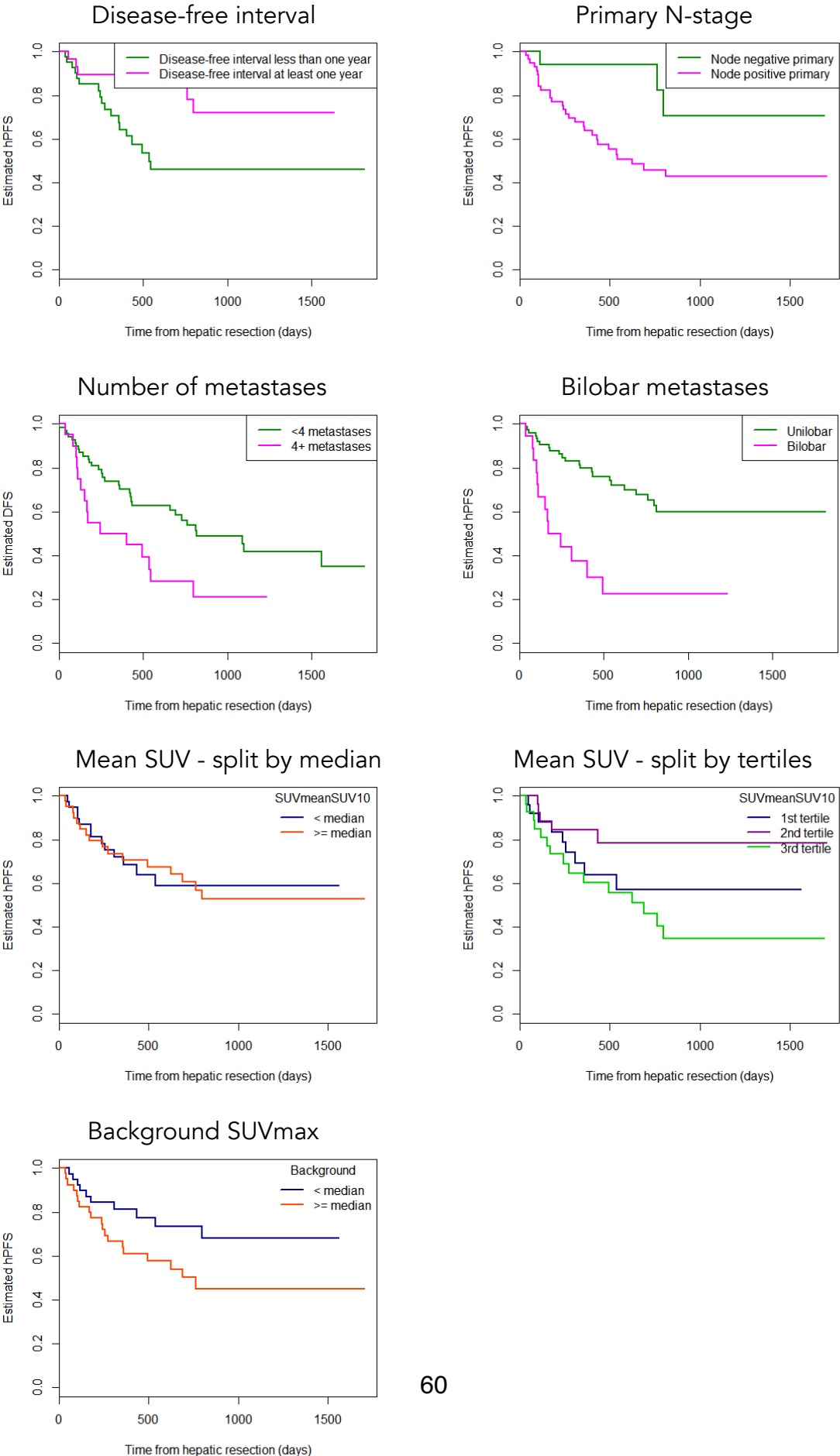


Table 3d Univariate analysis of variables for DFS and hPFS

Characteristic	DFS		hPFS	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Clinical variables				
Primary Tumour				
• Colon vs Rectum	0.86 (0.48-1.54)	0.616	0.78 (0.40-1.53)	0.475
• T-stage stage*	1.69 (0.98-2.91)	0.061	1.61 (0.89-2.98)	0.131
• T4 vs T1-3	5.69 (0.74-44.0)	0.095	3.89 (0.49-30.34)	0.197
• Node positive	2.56 (1.08-6.10)	0.033	3.62 (1.10-11.9)	0.034
DFI (years)%	0.73 (0.52-1.01)	0.061	0.59 (0.36-0.96)	0.034
Synchronous disease	1.27 (0.71-2.27)	0.425	1.56 (0.79-3.09)	0.197
Neoadjuvant chemotherapy	1.69 (0.92-3.10)	0.090	2.51 (1.18-5.35)	0.017
Conventional imaging variables				
Number of metastases#	1.15 (1.03-1.29)	0.016	1.25 (1.12-1.40)	<0.0001
Bilobar vs unilobar metastases	2.09 (1.06-4.13)	0.035	3.80 (1.91-7.56)	<0.001
Size of largest metastasis (cm)	0.92 (0.79-1.08)	0.306	0.96 (0.81-1.14)	0.638
Extrahepatic disease	2.98 (1.05-8.45)	0.040	2.20 (0.85-5.69)	0.103
¹⁸F-FDG-PET imaging variables				
Tumour PET features§				
• SUVmax	1.05 (0.80-1.39)	0.725	1.15 (0.84-1.59)	0.385
• SUVpeak	1.01 (0.74-1.38)	0.932	1.13 (0.79-1.62)	0.491
• SUVmean	1.22 (0.88-1.69)	0.224	1.41 (0.97-2.05)	0.068
• MTV	0.87 (0.92-1.21)	0.401	1.01 (0.75-1.36)	0.958
• TGVmax	0.83 (0.57-1.21)	0.327	0.95 (0.68-1.33)	0.783
• TGVmean	0.89 (0.65-1.22)	0.458	1.03 (0.78-1.37)	0.820
Background liver PET features§				
• SUVmax	1.13 (0.83-1.54)	0.451	1.25 (0.87-1.81)	0.232
• vs median	-	-	2.04 (0.95-4.39)	0.069
• Standard deviation SUV	1.21 (0.89-1.66)	0.228	1.23 (0.86-1.78)	0.258

Notes:

*HR for each increase in T-stage above T1

%HR for each disease-free year following resection of the primary

#HR for each additional metastasis >1

§HR for each standard deviation increase from mean

v. Multivariate analysis

The results of the multivariate analysis are shown in Table 3e. For DFS, increasing numbers of metastases, a node-positive primary tumour, higher primary tumour T-stage and greater SUVmean were predictors of poor outcome. In the model, those with smaller metastases did worse than those with larger metastases. For hPFS the number of metastases was a highly significant determinant of outcome, and T-stage was also a significant determinant. Primary tumour node positivity was a poor prognostic marker, left in due to its significance in earlier iterations in the model. Synchronous disease was a good prognostic marker in the model, also left in due to borderline significance during earlier iterations.

Table 3e: Multivariate model of variables for DFS and hPFS

Characteristic	HR (95% CI)	p-value
DFS		
Number of metastases [#]	1.399 (1.121-1.747)	0.003
Primary tumour node positive	1.852 (1.113-3.081)	0.018
Primary tumour T-stage*	2.49 (1.138-5.429)	0.022
Tumour SUVmean [§]	1.626 (1.016-2.604)	0.043
Size of largest metastasis (cm)	0.749 (0.565-0.994)	0.045
hPFS		
Number of metastases [#]	1.300 (1.135-1.490)	<0.001
Primary tumour T-stage*	2.469 (1.101-5.540)	0.028
Primary tumour node positive	1.300 (0.835-2.330)	0.204
Synchronous disease	0.537 (0.229-1.257)	0.152

Notes:

*HR for each increase in T-stage above T1. [#]HR for each additional metastasis >1

[§]HR for each standard deviation increase from mean

4. *Discussion*

There have been numerous studies examining risk-stratification of patients with oligometastatic CRC undergoing hepatic resection for survival or recurrence (Kanas et al., 2012). In this study of 96 patients we analysed the established imaging biomarkers of adverse outcomes: a greater number of metastases, larger metastasis(es), bilobar hepatic metastatic disease and EHD. We also analysed biomarkers derived from baseline ^{18}F -FDG-PET studies, including the routinely reported SUVmax, those that are not routinely reported derived from volumetric analysis of the PET data (SUVmean, MTV and TGV) and measurements of background liver FDG-activity and variability which have not previously been reported as biomarkers.

In this cohort we replicated established biomarkers for poor outcome; the T-stage (HR = 1.69) and nodal status (2.56) of the primary, a shorter DFI (0.73 for each additional disease free year), more numerous (1.15 for each additional metastasis) and bilobar (2.09) metastases, and EHD (2.98) were associated with decreased DFS. The order of magnitude of these effects was similar to those reported for OS. Not all of these variables were significant for hPFS. One observation was that the most significant risk factors for DFS were the number (1.25 for each additional metastasis) and bilobar distribution (3.80) of metastases, which appears to be more strongly associated with hPFS than DFS. The implication is that the burden

of previous disease spread to the liver confers a higher risk of developing future intrahepatic relapse, perhaps due to a more favourable microenvironment, or pre-metastatic niche (Peinado et al., 2017), given the characteristics of the tumour cells in circulation.

We also found that there were borderline significant associations of higher tumour SUVmean and higher background SUVmax with reduced hPFS. Higher SUVmean was also significantly associated with reduced DFS at multivariate analysis. Several studies have reported on pretreatment/baseline SUVmax as a prognostic biomarker, and have been subjected to meta-analysis (Xia et al., 2015). Pre-treatment SUV was a significant prognostic biomarker for OS in 3 of the 6 papers considered, and the overall effect was significant (HR = 1.24, $p = 0.008$). However, this analysis pooled disparate studies with different measures and thresholds of glucose uptake, and is probably not valid as a pooled result. Several of the studies are not referenced. In one study by de Geus-Oei et al. (2006) of 152 patients with resected mCRC, SUVmean, thresholded at the median value (SUVmean = 4.26), showed a significant difference in OS between groups (HR = 1.17, $p = 0.0002$). This was similar to the observed median SUVmean of 5.15 in our cohort. The size of the effect on hPFS in our study and OS in this study are of the same order of magnitude (1.17 vs 1.41) but are not directly comparable. Most other studies assessed SUVmax, which is more conventionally measured and typically reported in normal practice. For example Riedl et al. (2007), reported SUVmax was a poor prognostic marker for OS, using a range of thresholds, although none of the

patients in their study appeared to have been treated with neoadjuvant chemotherapy. An Australian group (Lau et al., 2014), reported on 80 patients, a similar proportion of whom were treated with neoadjuvant chemotherapy (63.8% compared 60.4% in our cohort), also did not find baseline SUVmax was a significant prognostic marker. Lau et al. also considered the volumetric ^{18}F -FDG-PET parameters MTV and TGV at baseline, again finding no association with outcome. These composite biomarkers for tumour burden and glucose consumption are not routinely measured, although they have been reported as prognostic biomarkers at baseline in other tumour such as pancreatic adenocarcinoma (Chirindel et al., 2015) and non-small cell lung cancer (Ohri et al., 2015).

^{18}F -FDG-PET/CT after neoadjuvant chemotherapy is not part of routine clinical practice in most UK institutions, and post-treatment imaging was available in only a small number of patients following chemotherapy, insufficient to analyse. Prior studies have reported that metabolic response to chemotherapy is an indicator of better prognosis, for examples change in SUVmax, MTV and TGV are reported to be prognostic marker in the setting of neoadjuvant chemotherapy for resected CRLM (Lau et al., 2014) and SIRT for CRLM (Gulec et al., 2011, Fendler et al., 2013). The SUVmax of the background liver parenchyma as a prognostic biomarker has not been previously reported. In this study, at univariate analysis, a SUVmax of the background higher than the median was estimated to double

recurrence risk compared with background SUVmax below the median, which was borderline significance.

There are several limitations to the presented analysis. This was a relatively small cohort of patients which in particular limited the power of the multivariate modelling to investigate multiple variables and interactions. Most previous studies have dealt with cohorts of greater than 100 resected patients, and sometimes over 1000 patients (Kanas et al., 2012; Table 1). Secondly, the clinical follow-up is short compared to others in this field (median follow-up 2 years). The gold-standard outcome measure in this setting would be OS, but this data was not available in most patients. OS is also affected by a wider number of clinical and patient variables than DFS, so may its use may have reduced the signal to detect effects of variables in this relatively small cohort. Two- or three-year DFS is a reasonable surrogate marker for OS in trials of adjuvant chemotherapy following curative-intent treatment in early CRC (Sargent et al., 2007), and this may also hold true when developing biomarkers for curative-intent treatment of oligometastatic CRC (Araujo, Herman & Riechelmann, 2017). In our study the median follow-up was 2 years, but a minimum follow-up of 2 years would be preferred. Hepatic PFS is an outcome measure adapted from trials of liver-directed therapy (e.g. Wasan et al., 2017). The rationale was that liver-specific progression is increasingly relevant in the evolving clinical management of liver-dominant disease, where the potential to prolong life is dependent on managing lifetime liver disease burden and function. This might inform the search for prognostic biomarkers in non-curative settings,

such as SIRT therapy or liver-targeted chemotherapy. Thirdly, this cohort is incompletely characterised for several factors that are known to have prognostic significance (Kanas et al., 2012), which undermines the strength of the multivariate analysis. The resection margin status was not included in the model. Involved (R1 and R2) surgical resection margins are associated with poorer outcomes and while not common, are a feature of a significant minority of colorectal hepatic metastasectomies (R1 resections approx. 8% and R2 resections approx. 9.1% in a large UK-based surgical series; Rees et al., 2008). The focus of the study was on imaging biomarkers and biomarkers available prior to intervention, it would have been helpful to correct for this in the multivariate analysis. Some of the variables related to the primary tumour were not available, such as the location and differentiation. While I distinguished between colon and rectal primaries, a more relevant division would have been into left and right sided tumours, as the molecular subtypes are differentially represented at different locations (Guinney et al., 2015) and have different biology (Ulivi et al., 2017) and outcomes (Ahmed et al., 2017). In this retrospective study, peri-operative CEA levels were available in few patients, reflecting the fact that this is not in routine use in clinical practice, although elevated CEA levels are known to confer worse prognosis. Similarly KRAS and BRAF mutations are known to confer worse outcomes (Tosi et al., 2017) but the mutation status was not routinely available, since it is not routine, or performed locally and not available in the tertiary surgical centre. Several other potential biomarkers, such as C-reactive protein (CRP) (Pathak et al., 2014), which might have been available, were not considered. In light of these limitations this

analysis would be best described as exploratory and would need investigation in a larger and more consistently described. However, it does suggest that biomarkers from baseline ^{18}F -FDG-PET might have additional value in risk stratifying patients with oligometastatic CRC prior to surgery. It also suggests that intrahepatic and extrahepatic recurrence might be usefully considered separately in terms of risk factors for recurrence.

In conclusion, conventional and ^{18}F -FDG-PET imaging variables were investigated as prognostic biomarkers in patients with resected CRLM, confirming the importance of the number and distribution of metastases, and the importance of accurate staging. Our data support prior studies suggesting that baseline higher tumour SUV might be associated with poorer outcomes and has identified background liver parenchymal FDG-uptake as a potential novel biomarker in this setting.

Chapter 4

Histopathological heterogeneity of CRLM

1. Introduction

Imaging techniques, whether morphological or functional, provide non-invasive assessment of tumour phenotypes. The imaging features observed is produced by the underlying structural or molecular phenotype of the tumour. For a given tumour type and location, these phenotypes vary between patients to different extents, depending on the characteristic being assessed; some features are relatively homogeneous, while other features take on a wider range of values. The

major advantage of imaging is that it is non-invasive, and therefore can be used to assess tumours *in vivo*, prior to any treatment decisions. However, particularly in the setting of metastatic cancer, which is often a multisite disease, a further advantage of imaging is that it can be used to assess tumours at multiple sites within a single patient. It is recognised that there is between and within patient tumour heterogeneity in patients with metastatic CRC, both in terms of genomic variation (e.g. Wei et al 2017) and in their histopathological features and immunohistochemical expression (Van den Eynden et al., 2012, Eefsen et al., 2012). In order to understand the potential role for imaging to assess the tumour phenotypes of CRLM, it is important to analyse the degree to which these phenotypes vary between and within patients. In the first part of this Chapter, I explore the variability of morphological histopathological features and protein expression in CRLM.

In the second part of this Chapter I present two pieces of lab-based work I performed to investigate features of the clinical assessments performed in this Chapter. Firstly, I used cell-culture techniques to assess differences in IHC expression and glucose utilisation and across CRC cell lines, to assess whether any heterogeneity observed *in vivo* may be related to tumour biology, rather than predominantly a feature of the tumour microenvironment. Finally, I began pilot work to look at whether DNA extracted from FFPE material could be used to examine patterns of chromosomal variation within and between tumours.

2. Chapter Methods

i. Patient cohort

As described in Chapter 2, these patients were drawn from a retrospective cohort of patients with CRLM who underwent surgical resection at a tertiary HPB centre.

ii. Histological assessment

The histopathological analysis is described in detail in Chapter 2. The slides were scored by two experienced consultant histopathologists (RG and LMW), who reviewed cases contemporaneously and reached an agreement for each feature. A thick pseudocapsule was defined as a collagen rim equal to or greater than 500 μm , and a thin pseudocapsule as less than 500 μm (Lunevicius et al., 2001). A thick capsule has been found to be prognostically relevant (Okano et al., 2000, Lucevicius et al., 2001) but a threshold based on a measurement, rather than assessment of the number of collagen fibres, was felt to be more easily reproducible. The lesions were categorised according to the original description by Vermeulen et al. (2001), which subdivides tumours into 'pushing', 'desmoplastic', including all lesions with a desmoplastic pseudocapsule, and 'replacement'. These classifications have been demonstrated to be prognostically relevant (Eefsen et al., 2015). Lesions were also categorised according to the subgroups described by Siriwardana et al. (2016), which subdivides tumours into 'encapsulated' (E) and 'non-encapsulated' (N). Encapsulated and desmoplastic are equivalent in these descriptions, and non-encapsulated encompasses both

pushing and replacement patterns. Again, this classification has been demonstrated to be prognostically relevant. Where more than one pattern was present, both were recorded but dominant pattern was used to determine the categorisation for that metastasis.

iii. Histological Analysis

The morphological features are presented on a per lesion basis in the tables below and summarised as overall frequencies. For patients with more than one metastasis, heterogeneity of a categorical feature was defined as where more than one categorical variable was present across all metastases in the patient. Population heterogeneity for each feature is the proportion of patients with heterogeneity.

As described, patients were considered to be high expressers for a given IHC marker if they were above the median value. For patients with more than one metastasis, heterogeneity of IHC expression was defined as where metastases with both high and low expression were present in one patient. Population heterogeneity for each marker is the proportion of patients with heterogeneous expression.

Patients were subdivided into those who had received neoadjuvant chemotherapy and those that had not and between-group comparisons performed using the Student T-test for continuous parametric variables and the

Mann-Whitney U Test for non-parametric variables. The Chi-squared or Fishers Exact Tests were used for categorical variables. Metastases were also subdivided into groupings described by Siriwardana et al (2016) i.e. those with pushing and replacement patterns were combined, and compared to those with desmoplastic patterns or desmoplastic capsules. The morphological features and IHC scores for those types of metastases were compared using the Student T-test for continuous parametric variables and the Mann-Whitney U Test for non-parametric variables. The Chi-squared or Fishers Exact Tests were used for categorical variables. P-values of <0.05 were used to determine significance. For comparisons of continuous variables, simple linear regression was performed.

iv. Cell Culture Experiments

Two dimensional cell culture

The following cell lines were cultured at 5% CO₂ humidified 37°C in Dulbecco Modified Eagle Medium (DMEM) (Gibco) with 10% Foetal Bovine Serum (FBS) (Gibco), penicillin-streptomycin (Gibco) and non-essential amino acids (Gibco, 5mL in 500ml) and passaged twice weekly:

- SW1222
- SW1463
- HT29
- HCT116

Cell pellets were made by detaching cells from the plates, followed by centrifuging the cells in an Eppendorf tube, washing (x3) with phosphate buffered

saline (PBS; Gibco) and then fixed in formalin for 24 hours, and then embedding in paraffin wax.

Spheroid culture

1000 cells from each cell line were plated on Corning Costar ultra-low adherence plates (Sigma-Aldrich), centrifuged and incubated at 5% CO₂ humidified 37°C in DMEM (Gibco) + 10% FBS (Gibco) + penicillin-Streptomycin (Gibco) + NEAA (Gibco, 5 mL in 500 mL) with the media changed every 48 hours. Once they had reached 600-1000 µm diameter, they were washed with PBS (Gibco), fixed in formalin for 24 hours, and then embedded, first in agarose gel to allow the small spheroids to be transferred to the cartridges, and then embedded in paraffin wax.

Immunohistochemistry

Cell pellets and spheroids were fixed in formalin for 24 hours and then embedded in paraffin wax. 5 µm slides were cut and stained as described in Methods. To confirm central hypoxia, pimonidazole was added to the culture medium overnight and spheroids were also stained for pimonidazole, again using the Leica BOND-MAX autostainer (Hypoxypoint, 1:1000, ER1(20), F30). The cell pellets were scored according to the proportion of cells stained by visual assessment. Spheroids were assessed visually to either show hypoxia-induced expression pattern (i.e. central necrosis, with rim of expression centrally and non-expression peripherally), its inverse (i.e. central necrosis, with rim of non-

expression centrally and expression peripherally), or a pattern not related to environmental hypoxia (random expression with respect to central necrosis).

24 hour glucose consumption experiment

The aim of this experiment was to measure the glucose consumed from culture medium by a known number of cells over 24 hours across colorectal cell lines using a colorimetric technique.

For each cell line, 40,000 cells (x3) were plated with 100 μ L DMEM 1 g/L in a 96-well plate and incubated for 24 hrs. To remove the cells, 75 μ L from each well was aspirated and spun for 10 mins at 8400 rpm then the supernatant was aspirated. The glucose concentration in the culture media was measured using the Sigma Glucose Assay Kit (GAGO-20, Sigma-Aldrich). Samples were diluted 1:20 with deionised water and aliquoted into wells. Each well contained 100 μ L assay + 50 μ L dilute sample. These were allowed to react for 30 minutes at 37 °C before stopping the reaction with 100 μ L H₂SO₄. Absorbance in each well was measured at 540 nm. The reference standard was calculated using the single sample method (Method 2) in the product documentation. Milligrams of glucose in each sample was calculated as per Method 2 and the glucose consumption calculated as a subtraction from the baseline glucose. This was completed in triplicate.

Glucose uptake experiment

The aim of this experiment was to measure the glucose taken up by a known number of cells from different cell colorectal cell lines by measuring the uptake of 2-Deoxy-D-glucose (2-DG) from buffer in 20 minutes using a colorimetric technique.

For each cell line 40,000 cells (x3) were plated with 100 μ L DMEM 1g/L in a 96-well plate and incubated for 24hrs to allow adherence. They were washed with PBS and then glucose-depleted for 1 hour in 100 μ L KRPH/2% BSA. The uptake experiment was performed as per the manufacturers instructions using the Abcam Glucose Uptake Assay Kit (ab136955). 10 mM 2-DG 10 μ L was added to the buffer for 20 minutes and then the cells were washed and then lysed. An nicotinamide adenine dinucleotide phosphate (NADP)-degradation step was performed to removed endogenous NADP. Cell lysate protein concentration was measured and standardised to 1 μ g protein in 50 μ L. The 2-Deoxyglucose-6-phosphate (2-DG6P) standard curve was prepared. The reaction for each sample was run as per the manufacturers instructions. Measurements were performed 5, 10 and 20 minutes after adding the final reaction agent using a microplate reader (POLARstar Omega, BMG Labtech) at 412 nm, and the absorbance at 20 minutes was used as the basis for the calculations as described in the assay protocol. This was completed in triplicate.

v. Genomic Experiments

Sample Selection

Experiment 1 – Growth Pattern.

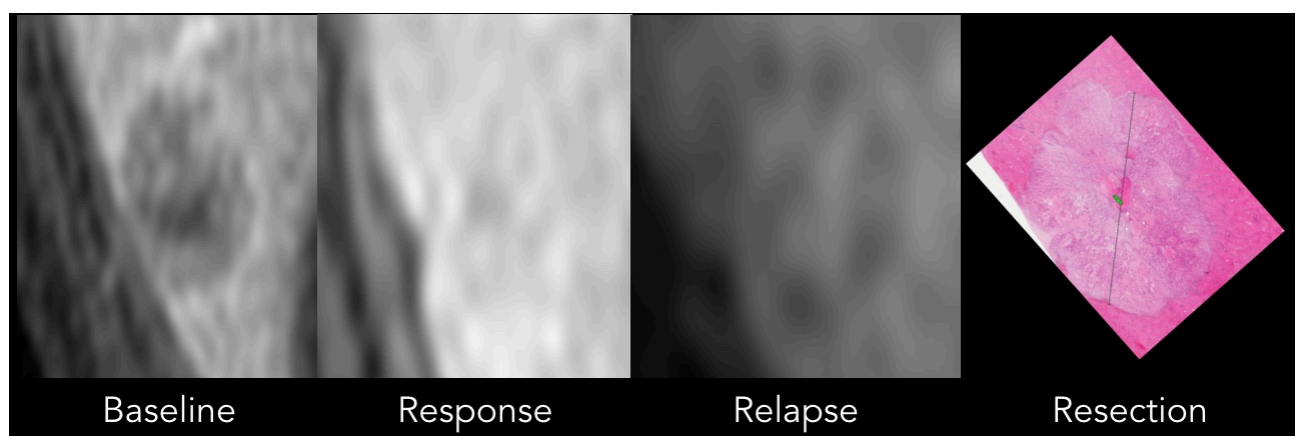
The aim of this experiment was to compare copy number aberrations (CNA) between different metastases with different (Vermeulen) patterns of tumour-liver interface. Seven metastases of metastatic subregions demonstrating each of the pushing, desmoplastic and replacement pattern of tumour-liver interface, as described above, were selected.

Experiment 2 – Clonal Evolution

The aim of this experiment was to demonstrate the feasibility of using imaging evidence of clonal evolution in CRLM following chemotherapy to select tumour subregions for DNA extraction and analyse CNA in these subregions. Evolution of chemotherapy resistance clones observed in two patients with CRLM treated with FOLFOX chemotherapy. These are characterised by initial response followed by 'budding' regrowth (Figure 4a). Both patient 1 (CRLM101) and patient 2 (CRLM87) had two metastases and several buds in one lesion.

Figure 4a: Clonal evolution on MRI after initial response to chemotherapy

The baseline post-contrast MRI demonstrates the hypo enhancing subcapsular metastatic deposit which then shows response to chemotherapy, reducing in size and with capsular retraction. After stopping chemotherapy, the tumour relapses with multiple hypenhancing tumour buds, which are visible in the resected specimen.



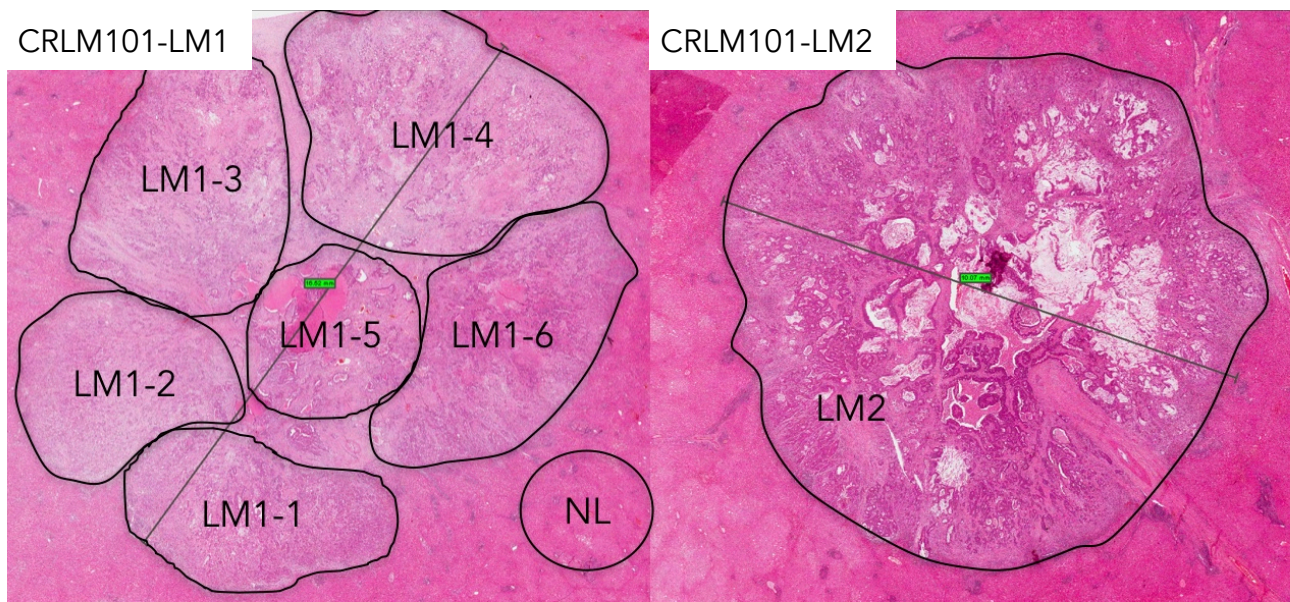
DNA Extraction

Sequential 5 µm FFPE slides of CRLM were used. A mask of the metastasis or metastatic subregion was delineated on an H&E stained slide (Figure 4b). Five sequential unstained slides were then dewaxed and placed on the mask one after another. The delineated tumour regions(s) were macrodissected, typically using a 21G or smaller needle to create a small pellet(s) which could be transferred into separate microcentrifuge tubes. DNA extraction was performed using the Qiagen QIAamp DNA FFPE Tissue Kit following the manufacturers instructions. Briefly, tissue is lysed using proteinase K, heated in appropriate buffers, and then DNA is

bound by passing through the QIAamp MinElute column, washed and then eluted into ATE buffer (10 mM Tris-Cl pH 8.3, 0.1 mM EDTA, 0.04% NaN₃ (sodium-azide)).

Figure 4b: Tumour masks for masks for FFPE macrodissection

These H&E stained slides show the extraction masks for Experiment 2 Patient 1 (CRLM101) showing the two extracted metastases (LM1 and LM2) from the same patient, with the subregions of LM1 selected for analysis.



DNA quantification

DNA quantification was performed using the PicoGreen dsDNA Assay Kit (Life Technologies) following the manufacturer's recommended protocol. aiming for at least 80 ng of genomic DNA. DNA quantification was performed both by myself and in house by Affymetrix.

OncoScan Assay

The OncoScan FFPE Assay Kit has been developed to generate genome-wide CNV and loss of heterozygosity (LOH), including 220,000 single nucleotide polymorphisms (SNPs) with high probe density covering ~900 cancer genes, as well as a limited panel of somatic mutations (74 mutations across 9 genes, BRAF, KRAS, EGFR, IDH1, IDH2, PTEN, PIK3CA, NRAS and TP53) from FFPE tissue (Foster et al., 2015). It is based on Molecular Inversion Probe technology (Hardenbol et al., 2003). Samples were analysed using the Affymetrix Research Services Laboratory as the OncoScan FFPE Express 2.0 Service (Affymetrix, Inc, Santa Clara, CA).

OncoScan Assay Analysis

Array fluorescence intensity data (CEL files), generated by Affymetrix GeneChip Command Console (AGCC) Software version 4.0 were processed using OncoScan Console software to produce OSCHP files and a set of quality control (QC) metrics. The QC metrics Median Absolute Pairwise Difference (MAPD) and normal diploid SNP Quality Control (ndSNPQC) are produced for each sample. Those satisfying $\text{MAPD} \leq 0.3$ and $\text{ndSNPQC} \geq 35$ are considered satisfactory and high quality, while those with $\text{MAPD} \leq 0.3$ and $\text{ndSNPQC} \geq 26$ but < 35 were considered satisfactory but borderline quality as previously described (Wood et al., 2017). The rest were considered unsatisfactory. Genomic copy number and aberrant cell fraction were calculated by the provided TuScan algorithm (Foster

et al., 2015). OSCHP files were viewed and analysed in the Nexus Express software.

The intention in Experiment 1 was to group patients by the Vermeulen pattern of tumour-liver interface, and look for common patterns of CNA between groups. Similarly, in Experiment 2 samples were to be grouped by patient and examine at commonalities and differences within and between metastases. These analyses were unfortunately not complete at the time of completing my CRF, and collaborators in the Wellcome Centre for Human Genetics, were no longer available to assist with the analysis. Therefore, only feasibility and preliminary descriptive data is presented.

3. *Results*

There were 76 metastases available for analysis in 45 patients. Of these patients, 25 (55.6%) were treated with neoadjuvant chemotherapy, affecting 49 (64.5%) metastases. One metastasis only had evidence of intravenous tumour, and therefore morphology could not be assessed. In seven metastases in four patients there were no viable tumour cells visible; these lesions were completely replaced by mucin, necrosis and fibrosis, or a combination. Of these patients, three had been treated with neoadjuvant chemotherapy. A summary of the features assessed is presented in Table 4a for all metastases, and subdivided by whether they were treated with neoadjuvant chemotherapy. Not all features were assessed in all lesions. The mean size was 30mm (range 4-150mm). The majority of tumours were moderately differentiated.

Table 4a: Histopathological features and IHC staining of CRLM

Feature	All lesions (n=76)	Chemotherapy (n=49)	No chemotherapy (n=27)	p-value
Mean size (mm; range)	30.0 (4-150)	27.8 (6-110)	33.0 (4-150)	0.427
Positive margin	0	0	0	1.000
Shape (%)				
• Round	24 (32.4)	17 (36.2)	7 (25.9)	0.290
• Lobulated	49 (66.2)	30 (63.8)	19 (70.3)	
• Irregular	1 (1.4)	0	1 (3.7)	
Differentiation (%)				
• Well	0	0	0	1.000
• Moderately	62 (92.5)	38 (92.7)	24 (92.3)	
• Poorly	5 (7.5)	3 (7.3)	2 (7.7)	
Mean percentage composition				
• Tumour cells	35.1	34.1	36.7	0.655
• Fibrosis	28.5	30.9	24.0	0.169
• Necrosis	28.5	25.0	34.6	0.133
• Mucin	7.9	9.9	4.4	0.323
Calcification (%)	22 (29.7)	14 (29.8)	8 (30.8)	1.000
TRG (%)				
• TRG1	6 (8.1)	5 (10.6)	1 (3.7)	0.191#
• TRG2	6 (8.1)	5 (10.6)	1 (3.7)	
• TRG3	24 (32.4)	16 (34.0)	8 (29.6)	
• TRG4	28 (37.8)	17 (36.2)	11 (40.7)	
• TRG5	10 (13.5)	4 (8.5)	6 (22.2)	
Capsule (%)				
• None	49 (65.3)	33 (68.8)	16 (59.3)	0.455
• Any	26 (34.7)	15 (31.2)	11 (40.7)	
• Thin (<500 µm)	23	13	10	
• Thick (>500 µm)	3	2	1	
Capsule thickness (µm; range)	41.0 (0-585)	20.4 (0-500)	77.5 (0-585)	0.109
Compressed peripheral hepatocytes				
• None	21 (29.2)	13 (28.3)	8 (30.8)	1.000
• Any	51 (70.8)	33 (71.7)	18 (69.2)	
Mean compressed peripheral hepatocytes (µm; range)	80.1 (0-1924)	66.7 (0-1924)	103.7 (0-1791)	0.646
Vermeulen Pattern (%)				
• Pushing	27 (39.7)	17 (40.5)	10 (38.5)	0.985
• Desmoplastic	28 (41.2)	17 (40.5)	11 (42.3)	
• Replacement	13 (19.1)	8 (19.0)	5 (19.2)	
Siriwardana Pattern (%)				
• Encapsulated	28 (41.2)	17 (40.5)	11 (42.3)	1.000
• Non-encapsulated	40 (58.8)	25 (59.5)	15 (57.7)	
Local invasion (%)				
• Biliary	2 (2.8)	1 (2.2)	1 (3.8)	1.000
• Venous	21 (29.2)	14 (30.4)	7 (26.9)	0.794
• Perineural	2 (2.8)	1 (2.2)	1 (3.8)	1.000
ki67 (median and range)	70 (0-90)	70 (0-90)	70 (0-90)	0.656
CA9 (median and range)	40 (0-100)	50 (0-100)	40 (0-100)	0.379
GLUT1 (median and range)	40 (0-95)	40 (0-90)	35 (0-95)	0.503
Notes: #TRG1-2 vs TRG3-5				

i. Morphological features

Tumour components and viability

The proportion of viable tumour varied considerably from lesion-to-lesion, from no viable tumour cells to 90% viability. The proportion of viable cells did not appear to be affected by neoadjuvant chemotherapy (34.1% vs 36.7%; $p=0.655$). Similarly, the proportion of fibrosis and necrosis varied from zero to 95% and 90% respectively. There was a greater proportion of fibrosis and TRG1-2 metastases in the treated group (21.3% vs 7.4%, $p=0.191$), which might be expected since treatment may induce tumour response with fibrosis, but this was non-significant.

Vascular, perineural and biliary invasion

Vascular invasion was present in 21 (29.2%) metastases, which was exclusively due to portal venous invasion; there were no cases of arterial invasion in this series. These were similarly distributed between encapsulated and non-encapsulated metastases (11/28 vs 10/40; $p=0.453$) and between the Vermeulen patterns. Perineural and biliary invasion were rare, each observed in only two metastases.

Tumour-liver interface

The tumour liver interface was assessed according to the categorisations proposed by Vermeulen et al. (2001) and Siriwardana et al. (2016) and these are summarised in Table 4b. There were 27 (39.7%) pushing, 28 (41.2%) desmoplastic (including pushing metastases with desmoplastic capsules) and 13 (19.1%) replacement. 5.3% were mixed, but were categorised as the predominant pattern.

In none of the metastases did the secondary pattern occupy greater than 25% of the interface. For the 17 patients with more than one assessable metastasis, 8 (47.1%) were heterogenous for the Vermeulen pattern between metastases, of which 6 were due to the presence of pushing and replacement patterns in two metastases or more metastases in the same patient. There were 28 (41.2%) encapsulated and 40 (58.8%) non-encapsulated metastases For the 17 patients with more than one assessable metastasis, 2 (11.8%) were heterogenous for the Siriwardana pattern between metastases.

Table 4b: Patterns of tumour-liver interface in individual metastases

Patient	# metastases assessed	Vermeulen Pattern [§]	Heterogenous	Siriwardana Pattern [®]	Heterogeneous
CRLM002	3	D, D, D*	No	E, E, E	No
CRLM004	5	P, D*, P, R, D*	Yes	N, E, N, N, E	Yes
CRLM005	1	D*	.	E	.
CRLM016	1	P	.	N	.
CRLM017	2	P, R	Yes	N, N	No
CRLM019	3	NT, NT, NT	.	NT, NT, NT	.
CRLM021	4	R, R, P, P	Yes	N, N, N, N	No
CRLM023	1	D*	.	E	.
CRLM024	1	R	.	N	.
CRLM025	1	P	.	N	.
CRLM028	3	R, P, P	Yes	N, N, N	No
CRLM030	1	D*	.	E	.
CRLM031	2	P, P	No	N, N	No
CRLM034	1	DD*	.	E	.
CRLM038	2	P, PR	No	N, N	No
CRLM040	2	D*, IV	.	E, IV	.
CRLM041	2	DD*, D*	No	E, E	No
CRLM047	2	P, P	No	N, N	No
CRLM048	2	DD*, P	Yes	E, N	Yes
CRLM049	3	D*, D*, D*	No	E, E, E	No

Patient	# metastases assessed	Vermeulen Pattern [§]	Heterogenous	Siriwardana Pattern [@]	Heterogeneous
CRLM050	1	R	.	N	.
CRLM051	1	P	.	N	.
CRLM052	2	D*, D*	No	E, E	No
CRLM053	2	R, P	Yes	N, N	No
CRLM055	4	R, P, R, P	Yes	N, N, N, N	No
CRLM062	1	D*	.	E	.
CRLM067	1	D*	.	E	.
CRLM069	1	NT	.	NT	.
CRLM071	1	R	.	N	.
CRLM073	2	P, P	No	N, N	No
CRLM076	1	NT	.	NT	.
CRLM077	2	NT, NT	.	NT, NT	.
CRLM086	1	D	.	E	.
CRLM087	2	P, R	Yes	N, N	No
FILM001	2	P, P	No	N, N	No
FILM002	1	D*	.	E	.
FILM003	1	R	.	N	.
FILM004	1	D*	.	E	.
FILM005	1	P	.	N	.
FILM006	1	D*	.	E	.
FILM008	1	D*	.	E	.
FILM010	1	D	.	E	.
FILM011	1	D*	.	E	.
FILM013	1	P	.	N	.
FILM015	1	D	.	E	.
Total (%)	76		8 (47.1) ^{&}		2 (11.8) ^{&}
	Pushing	27 (39.7) [#]	Encapsulated	28 (41.2) [#]	
	Desmoplastic	28 (41.2) [#]	Non-encapsulated	40 (58.8) [#]	
	Replacement	13 (19.1) [#]			

Notes

NT = no tumour, IV = intravenous tumour only

[§]Vermeulen Patterns: P = pushing pattern, D = desmoplastic pattern, D* = pushing pattern with desmoplastic capsule, R = replacement pattern. Two letters indicates mixed pattern; first letter dominant.

[@]Siriwardana Patterns: E = encapsulated, N = non-encapsulated

[&]Patients with at least two metastases and excluding metastases with no tumour

[#]Excluding metastases with no tumour

ii. Immunohistochemical staining

Immunohistochemical assessment was performed in 67 of 76 metastases; 9 patients had no viable tumour for scoring, and one metastasis was not analysed. The results are summarised in Table 4c.

ki67

The median expression of ki67 was 70% of cells and the range was 0-90%. The median was used to subdivide metastases into high and low expressers. There was no significant difference in ki67 expression between patients who had been treated with neoadjuvant chemotherapy and those that had not ($p>0.1$). There was also no difference between encapsulated and non-encapsulated metastases ($p>0.1$), or between different Vermeulen patterns of tumour liver-interface.

Of 18 patients with greater than one metastasis, there were both high and low expressers within a single patient in 15 (83.3%) patients.

CA9

The median expression of CA9 was 40% of cells and the range was 0-100%. The median was used to subdivide metastases into high and low expressers. There was no difference in CA9 expression between patients who had been treated with neoadjuvant chemotherapy and those that had not ($p>0.1$). Although the CA9 expression was higher in encapsulated than non-encapsulated metastases, this was non-significant (50.7% vs 41.9%, $p=0.250$), even when considering only the

untreated subgroup (49.5% vs 33.0%, $p=0.242$), or between different Vermeulen patterns of tumour liver-interface.

Of 18 patients with greater than one metastasis, there were both high and low expressers within a single patient in 6 (33.3%) patients.

GLUT1

The population median expression of GLUT1 was 40% of cells and the range was 0-95%. The median was used to subdivide metastases into high and low expressers. There was no significant difference in GLUT1 expression between patients who had been treated with neoadjuvant chemotherapy and those that had not ($p>0.1$). The GLUT1 expression of encapsulated metastases was significantly higher than non-encapsulated metastases (49.8% vs 32.69%; $p=0.016$). Similarly, desmoplastic metastases had a higher GLUT1 expression than pushing metastases (49.8% vs 29.3%; $p=0.009$).

Of 18 patients with greater than one metastasis, there were both high and low expressers within a single patient in 10 (55.6%) patients.

Table 4c: IHC expression within and between patients with CRLM

Patient	#	ki67						CA9						GLUT1						
		Met	1	2	3	4	5	Het	1	2	3	4	5	Het	1	2	3	4	5	Het
CRLM002	3	40	80	40				Yes	30	90	50			Yes	20	70	80			Yes
CRLM004	5	80	60	60	40	60		Yes	20	30	60	10	20	Yes	5	5	30	0	30	No
CRLM005	1	80							50						10					
CRLM016	1	80							40						50					
CRLM017	2	50	70					Yes	50	70				No	75	50				No
CRLM019	3	NT	NT	NT					NT	NT	NT				NT	NT	NT			
CRLM021	4	30	70	70	60			Yes	50	60	100	80		No	50	30	5	50		Yes
CRLM023	1	60							70						70					
CRLM024	1	70							40						30					
CRLM025	1	90							90						20					
CRLM028	3	60	70	70				Yes	40	0	10			Yes	80	0	90			Yes
CRLM030	1	10							90						90					
CRLM031	2	90	60					Yes	60	60				No	15	50				Yes
CRLM034	1	70							30						70					
CRLM038	2	90	60					Yes	0	30				No	0	10				No
CRLM040	2	90	10					Yes	100	20				Yes	50	40				No
CRLM041	2	70	50					Yes	80	100				No	95	90				No
CRLM047	2	80	50					Yes	10	30				No	10	80				Yes
CRLM048	2	90	60					Yes	30	20				No	20	60				Yes
CRLM049	3	70	50	60				Yes	20	5	50			Yes	90	30	50			Yes
CRLM050	1	90							10						75					
CRLM051	1	60							30						10					
CRLM052	2	80	60					Yes	70	60				No	10	30				No
CRLM053	2	70	90					No	30	10				No	80	0				Yes
CRLM055	4	90	0	10	40			Yes	80	15	60	30		Yes	40	5	5	40		Yes
CRLM062	1	NT							NT						NT					
CRLM067	1	90							30						50					
CRLM069	1	NT							NT						NT					
CRLM071	1																			
CRLM073	2	90	90					No	70	80				No	5	60				Yes
CRLM076	1	NT							NT						NT					
CRLM077	2	NT	NT						NT	NT					NT					
CRLM086	1	80							50						70					
CRLM087	2	60	90					Yes	20	0				No	30	5				No
FILM001	2	80	70					No	70	40				No	20	10				No
FILM002	1	60							80						60					
FILM003	1	80							100						40					
FILM004	1	80							60						40					
FILM005	1	60							40						10					
FILM006	1	90							30						40					
FILM008	1	60							10						50					
FILM010	1	10							90						5					
FILM011	1	20							5						60					
FILM013	1	70							20						50					
FILM015	1	90							40						60					
Total	76							15						6						10
(%)*								(83.3)						(33.3)						(55.5)

Notes

= number of metastases

Het = patient heterogeneous for IHC marker expression where more than two metastases.

* Patients with at least two metastases and excluding metastases with no tumour

iii. Cell culture experiments

Two-dimensional cell culture

Cell culture and IHC of cell pellets was successful in all cases. All cell lines showed 100% staining for ki67, indicating expected ongoing replication throughout the 2D culture. GLUT1 staining in normoxic, high glucose culture conditions was highly variable (Table 4d).

Table 4d: IHC staining of cell pellets of colorectal cell lines (% staining cells)

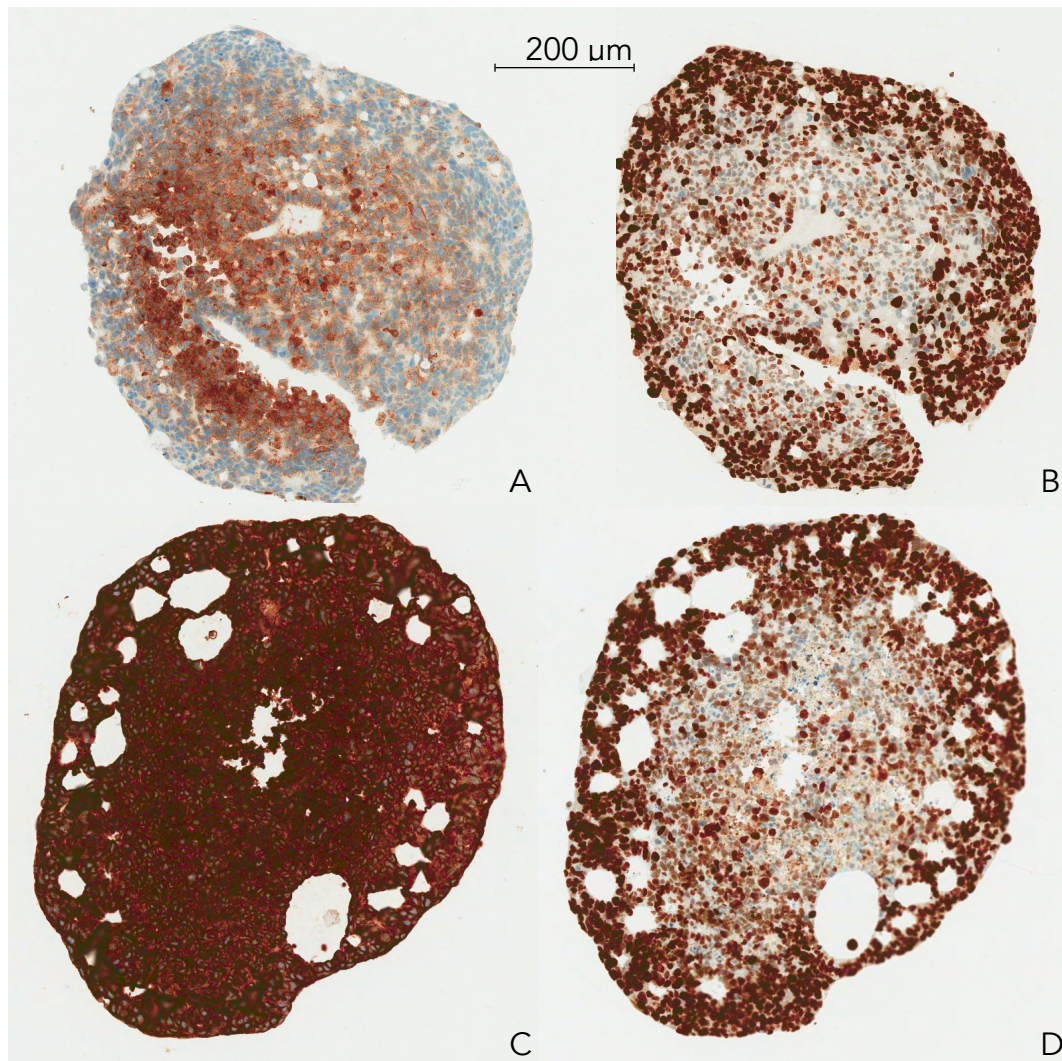
	SW1222	SW1463	HCT116	HT29
ki67	100	100	100	100
GLUT1	1	50	10	100

Spheroid culture

SW1463 did not adhere into spheroids. SW1222 and HCT116 formed tight spheroids with a hypoxia-induced expression pattern of GLUT1, and an inverse pattern of ki67 expression, while HT29 continued to express GLUT1 throughout, despite central necrosis and an inverse pattern of ki67 expression (Figure 4c).

Figure 4c: GLUT1 and ki67 staining of SW1222 and HT29 spheroids

Image A and B show a spheroid of SW1222 cells stained for GLUT1 and ki67 ,respectively. Image C and D show a spheroid of HT29 cells stained for GLUT1 and ki67, respectively. SW1222 which had no staining for GLUT1 at in 2D culture had increased GLUT1 expression, and reduced ki67 expression, centrally, presumably in response to hypoxia. HT29, conversely, retains complete GLUT1 staining demonstrated in 2D culture but again demonstrates central reduced ki67 expression in response to hypoxia.



Glucose depletion and uptake assays

The low GLUT1 expressing cell line, SW1222, had the lowest glucose uptake by both methods, and the high GLUT1 expressing cell line, HT29, had the highest glucose uptake, while SW1463 and HCT116 returned intermediate values (Table 4e).

Table 4e: Glucose consumption and uptake experiments

		Cell line			
24-hour glucose consumption (mg)	Run	SW1222	SW1463	HCT116	HT29
	1	0.348	0.468	0.517	0.501
	2	0.113	0.488	0.672	0.577
	3	0.244	0.49	0.559	0.528
	Av	0.235	0.482	0.583	0.535
	SD	0.118	0.012	0.080	0.039
20-minute Glucose Uptake (pmol/mL)	1	39.489	89.22	70.259	156.591
	2	10	60.784	83.333	130.196
	3	22.549	72.353	53.137	95.882
	Av	24.013	74.119	68.910	127.556
	SD	14.799	14.300	15.143	30.440

iv. Genomic Experiments

The DNA yield was sufficient in 26 of 27 (96.3%) samples. The sample with suboptimal DNA yield was of a small (<5 mm) subregion of a metastasis selected because of its different pattern of tumour-liver interface compared with the remainder of the metastasis. 21 of 27 (77.8%) samples were within the MAPD target and 18 of 27 (66.7%) samples were within the ndSNPQC target. Overall,

one third (9/27) of samples were high quality, one third (9/27) were borderline quality and one third (9/27) were unsatisfactory (Table 4f).

There were three different TP53 mutations demonstrated in nine samples in five patients. One mutation of TP53 (p.R306*:c.916C>T) was detected in all five samples from CRLM87. There were two different KRAS mutations demonstrated three samples in two patients. One mutation of KRAS (p.G13D:c.38G>A) was detected in both samples from CRLM34. There was one mutation of the PI3KCA gene.

Despite only small tumour subregions of 5 µm FFPE slides being harvested, all the 10 samples used for the clonal evolution projected had sufficient quantity and quality of material. The overall CNA (gains and losses) in these ten samples is shown in Figure 4d. This shows expected predominant within-patient concordance of CNA the tumour, given the same tumour origin. Patient 1 LM1-1 through LM1-4 had significant commonality in their CNA, whereas there were differences between LM1 subpopulations and LM2, for example the losses in chromosomes 3p and 4p and the gain in 6q were not present in LM2. Similarly, in Patient 2, LM1-1 through LM1-3 had significantly commonality in their CNA, whereas there were differences between these subpopulations and LM2, for example absent losses in 1p, 14q, 18 and 22 and gains in 2p, 3, 4, 5p, 6, 9, 12q. Interestingly, a CNA present in LM2 had been acquired in LM1-3 (gain in chromosome 13) and numerous CNA were common to LM1-4 and LM2.

Figure 4d: OncoScan OSCHP data viewed using Nexus Express

Copy number aberrations (gains in blue, losses in red) displayed by chromosome region (x-axis) in ten samples from two metastases (LM1 and LM2) from two patients, grouped by patient.

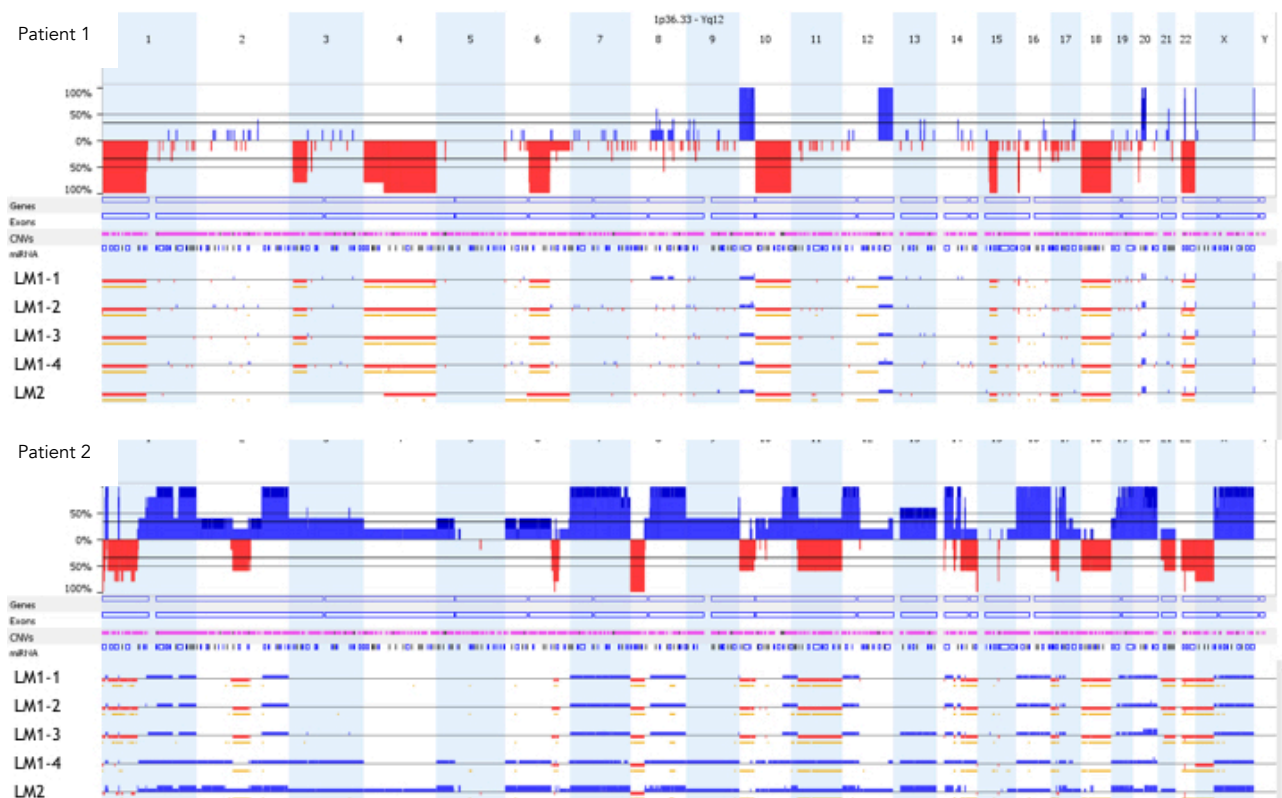


Table 4f: DNA yield, total copy number aberrations, percentage loss of

Sample Name	Internal Pico (ng/μl)	Internal Total DNA (ng)	Total CN aberrations	% LOH	MAPD	ndSNPQC
C05-2	13.9	1195.74	178	13	0.25	41.2
C21-1	16.16	1389.85	67	35.6	0.31	18.4
C21-4	16.63	1430.46	125	31.8	0.33	15.9
C31-1	5.48	471.02	85	18.3	0.28	23.1
C34-1-1	3.1	266.57	144	49.2	0.29	9.69
C34-1-2	0.67	57.41	92	38	0.38	16.4
C38-1	23.27	2001.07	72	10.5	0.26	28
C50-1	13.29	1089.66	116	34.8	0.31	17.2
C53-1	16.4	1344.74	99	61.6	0.33	15.6
C55-1	38.24	3135.41	124	17.5	0.25	28.8
C71-1	25.53	2093.75	177	20.6	0.29	24.9
C102-1	38.3	3140.89	118	13.3	0.27	32.7
C103-1	21.28	1744.71	117	27.3	0.26	36.3
C104-1	24.53	2011.38	88	38.2	0.26	36.6
C106-1	36.06	2956.65	147	30.6	0.26	33.6
C108-1	28.43	2330.93	167	18.9	0.44	10.2
C108-2	4.08	334.83	75	23.4	0.25	34.7
C101-1-1	1.77	144.83	66	22.3	0.27	35
C101-1-3	4.27	367.01	83	22.4	0.24	43.8
C101-1-4	2.56	220.48	46	22.3	0.25	42.3
C101-1-6	1.39	119.95	83	22.4	0.27	36.1
C101-2	14.68	1262.89	44	20.3	0.26	33.6
C87-1-1	6.8	585.1	142	20.9	0.24	35.2
C87-1-2	6.58	566.14	130	21.5	0.24	34.8
C87-1-3	2.75	236.54	150	20.8	0.25	36
C87-1-4	9.69	833.43	166	18.6	0.25	29.9
C87-2	23.7	2038.14	196	17.3	0.25	31.7
Notes Internal = DNA quantification performed by Affymetrix % LOH = percentage loss of heterozygosity MAPD = Median Absolute Pairwise Difference (target < 0.3; indicated in green) ndSNPQC = normal diploid SNP Quality Control (high quality > 35, indicated in green; borderline 26-35, indicated in yellow)						

Discussion

In this Chapter a population of resected CRLM are characterised in detail according to pre-defined morphological criteria and protein expression for markers of proliferation and hypoxia.

Similar to several prior studies, there is significant variability in the composition of CRLM at baseline and after neoadjuvant chemotherapy. The proportion of viable tumour and necrosis in particular has little relation to the treatment status. Some authors have distinguished between treatment-related 'infarct-like' and 'usual' necrosis (Li Chang et al., 2012, Ishidia et al., 2015), but we did not distinguish between these types. There were higher proportions of fibrosis (30.9% vs 24.0%) and a higher proportion of 'good' tumour regression grades (TRG1-2; 21.2% vs 7.4%) in the treated group, although these differences were non-significant. This is compatible with the findings of a larger series of treated metastases (Rubbia-Brandt et al., 2007), and would be consistent with predominantly fibrotic response to neoadjuvant chemotherapy in CRLM.

Venous invasion is a common features of CRLM; in our series it was present in 29.2% of metastases. Other studies have reported it in 10-49%, typically present in around a quarter of patients, so the present series is entirely consistent. In CRC, vascular invasion of the primary tumour has been associated with adverse outcomes (Talbot et al., 1980, Koukourakis et al 2005, Morris et al., 2006), related

to increased incidence of subsequent metastases. Similarly in CRLM, venous invasion has been associated with adverse outcomes (Yamamoto et al., 1999, Hayashi et al., 2010). In primary rectal cancer, the presence of EMVI can be demonstrated by MRI (Brown et al., 2003), and mrEMVI has been subsequently demonstrated to be a prognostic biomarker in its own right (Hunter et al., 2012). Given the prevalence of venous invasion in CRLM, developing mrEMVI as a biomarker for CRLM may be clinically useful.

There is growing interest in the pattern of tumour-liver interface in CRLM. The growth pattern provides an indication of the underlying tumour biology, which has implications for prognosis and therapy. Pushing patterns have been characterised as having angiogenesis dependent growth while the replacement pattern is non-angiogenic (Eefsen et al., 2012), and the latter respond poorly to anti-angiogenic agents (Frentzas et al., 2016). Encapsulated or desmoplastic metastases have improved outcomes compared with non-encapsulated metastases (Eefsen et al., 2015; Siriwardana et al., 2016). The distribution of metastases demonstrating pushing, desmoplastic and replacement patterns in this series, 39.7%, 41.2% and 19.1% respectively, is similar to that reported in other series. For example, Eefsen et al (2015) reported 44.0% pushing, 36.3% desmoplastic, 19.7% replacement (excluding 16.1% mixed) in a large series of CRLM. Our series had a preponderance of non-encapsulated (58.8%) over with non-encapsulated (41.2%) metastases compared with the small series from Siriwardana et al (2016), although, this is more consistent with other series (van de

Eynden et al., 2012; Eefsen et al., 2015). As in prior series, we found no effect of chemotherapy on the prevalence of each of the growth patterns.

Only one group has documented on the heterogeneity of growth patterns where there are multiple metastases (Eefsen et al., 2012; Eefsen et al., 2015), describing between-lesion heterogeneity in 20% and 62.6% of patients, which is comparable to 47.1% we have reported. The between-lesion heterogeneity of the two Siriwardana patterns, encapsulated and non-encapsulated, was much lower (11.8%). This was because pushing and replacement patterns co-existed in the majority (75%) of patients when between-lesion heterogeneity of growth patterns was observed. As in the study by Eefsen et al., (2012), true desmoplastic patterns and replacement patterns do not seem to co-exist. Based on a similar observation previously, it has been suggested that the replacement pattern may be a slower growing subset of the pushing pattern (van den Eynden et al., 2012).

Siriwardana et al., (2016) reported higher levels of the hypoxia-related factors HIF-1a and CA9 in untreated encapsulated metastases. In our series, although CA9 expression was higher in the encapsulated group, and the absolute difference greater when considering the untreated subgroup, neither comparison was significant. However, GLUT1, which is upregulated by HIF-1a, was found to be significantly higher in encapsulated metastases, which would corroborate their finding that encapsulated metastases are more hypoxic. It has been suggested that intratumoral hypoxia in encapsulated tumours may be caused by the fibrous

capsule impeding the recruitment of new vessels from surrounding normal liver parenchyma (Siriwardana et al., 2016). At least a proportion of CRLM and liver metastases from other primary sites gain their blood supply by vascular co-option from the liver parenchyma (Vermeulen et al., 2001; Donnem et al., 2013). A desmoplastic reaction to a liver metastasis may impair this process, making them reliant on neoangiogenesis, suggested by higher VEGF expression reported in this type of metastasis (Siriwardana et al., 2016).

In our study, ki67, GLUT1 and CA9 expression were heterogeneous in 83.%, 55.6% and 27.8% of patients with two or greater metastases respectively. This degree of variability has not been previously described and has potentially important implications. Heterogeneity of ki67 may indicate that different metastases are proliferating at different rates in response to a combination of tumour biology, local microenvironment and chemotherapy. CA9 and GLUT heterogeneity, despite limited evidence for heterogeneity of encapsulated/non-encapsulated metastases, suggests that the tumour microenvironment at least partially dictates which metastases become hypoxic. GLUT1 heterogeneity has implications for clinical imaging. ^{18}F -FDG-PET/CT is routinely used in staging resectable CRLM and this test is reliant on increased uptake of glucose in many solid organ tumours. GLUT1 is an important upregulated glucose transporter in many tumours including CRC (Younes, Lechago and Lechago, 1996; Hong and Lim, 2012). If it is heterogeneously expressed in metastatic populations, this may limit the sensitivity of ^{18}F -FDG-PET, particularly on a per-lesion basis.

The majority of the histological and immunohistochemical features discussed in this chapter have been found to be prognostically important in patients with resectable CRLM. However, between-lesion, within-patient heterogeneity is common, presumably due to a complex interplay between variations in the underlying genomics of tumour subclones and the metastatic niche, which governs the resulting tumour phenotype. Therefore, for these proposed biomarkers to be clinically relevant, for example to assist in prognostication or treatment selection, they must be reliably determined *in vivo* and potentially across all metastatic sites. This is particularly relevant in the palliative treatment setting where response to, for example, antiangiogenic agents may be determined by the growth pattern (Frentzas et al., 2016). It is not practically feasible or safe to biopsy multiple metastatic sites, and therefore non-invasive techniques are required to allow full characterisation of the tumour phenotype(s).

There are some limitations of the analysis performed. Firstly, the cohort of patients is moderate in size compared with the literature, and the subset with more than two metastases is not as large as prior series. Approximately half of patients received neoadjuvant chemotherapy. This limits the potential to analyse subgroups of treatment naive patients. Secondly, liver metastases are 3D structures and these assessments were carried out at a single level, in 2D. It is likely, therefore that the degree of heterogeneity within each lesion, for example the proportion of mixed growth patterns, is underestimated by this and other

studies. Careful prospective specimen handling, fixation and sectioning would be required to improve coverage of the entire lesion. For the majority of features I did not attempt to assess intra-lesional heterogeneity, which is a recognised feature of individual tumours. While potentially important, the relatively small dataset meant that analysis had to be relatively simplistic, with single scores attached to each feature. Secondly, the overall aim of this thesis is to investigate potential imaging, rather than histopathological or immunohistochemical, biomarkers. The spatial resolution of imaging techniques is likely to preclude assessment of within-lesion variation of many of these features.

The cell culture experiments showed that colorectal cell lines have heterogeneous GLUT1 expression in normoxic, non glucose-deprived conditions, and GLUT1 expression can be induced in response to hypoxia in cell lines that normally have low GLUT1 expression. However, GLUT1 expression does not necessarily imply hypoxia. Glucose uptake was related to GLUT1 expression, which would be expected as GLUT1 is the primary glucose transporter in CRC cells (Haber et al., 1998). These findings have implications for the use of ^{18}F -FDG-PET as a biomarker in CRC and colorectal oligometases. Some metastasising cells may have become Warburg adapted, and therefore have constitutively unregulated glucose transporters in normoxic conditions, while other cells may be upregulating GLUT1 in response to environmental hypoxia in situ. These different processes were observed in these experiments and will both cause increased GLUT1 expression and therefore glucose (and FDG) uptake, but may have

different implications for treatment response and prognosis. Biomarkers derived from ^{18}F -FDG-PET may be unable to disambiguate these processes, and it may be necessary to divide patients on the basis of tissue characteristics such as KRAS mutation status (Yun et al., 2009), or used in combination with other imaging techniques that estimate perfusion or hypoxia, as has been suggested previously (Miles and Williams, 2008).

Towards the end of this project I explored the feasibility of extracting DNA from FFPE samples. Although DNA quality is degraded in FFPE tissue when compared with fresh frozen material, the tissue can be extracted with preserved of spatial information, and allowed me to adopt the novel approach of using imaging features to guide macrodissection. More robust techniques for analysing FFPE tissue are now being developed, and most of the tissue extracted, was of sufficient quality and quantity to analyse SCNAs using a MIP technique, despite selective extraction from small tumour subregions in some cases. Preliminary analysis of subregions has confirmed commonalities and discrepancies in the chromosomal landscape of CRLM, both between and within individual metastases, which warrant further exploration. Analysis of within and between patient variation in the clonal evolution study is ongoing, as is between-group comparison of the histological phenotypes.

In conclusion, there is substantial between- and within-patient inter-lesional variation of clinically relevant morphological features and protein expression. of

These biomarkers have been described in *ex vivo* specimens following resection. This limits their clinical utility to the adjuvant setting. For them to become useful biomarkers to guide treatment before liver-directed therapy, or to be used in the larger population of patients with non-resectable CRLM, they need to be assessable before treatment is offered. Furthermore, variations in observed tumour phenotype will be due to a combination of variations in underlying genetic differences in tumours observed between and within patients, and the tumour microenvironment. Nice multisite or repeated percutaneous biopsy is neither clinically practical or safe, morphological and functional imaging techniques, which can non-invasively assess multisite disease, could be used to assess these tumour phenotypes *in vivo*. Composite or multiparametric imaging techniques may be required to unpick the multifaceted interaction of tumour biology and microenvironment. .

Chapter 5

Morphological and functional imaging of CRLM

1. *Introduction*

As set out in Chapter 1, there are clinically relevant histopathological features of CRLM which can be assessed in resection specimens in patients undergoing metastasectomy. These histopathological biomarkers are of limited value in determining patient management because they can only be assessed *ex vivo* i.e. after the decision has been made to offer radical treatment, and can only be assessed in the small subset of patients who are managed surgically. *In vivo* assessment may potentially be able to support clinical decision-making before

therapeutic intervention. Imaging phenotypes observed at conventional cross-sectional imaging are the product of the structural histopathological features and tissue composition. Morphological tumour features have been successfully evaluated and are used in clinical practice to assess prognostically relevant morphological tumour features, for example T-stage and EMVI status in primary rectal cancer (Hunter et al., 2011). Modern multiparametric liver MRI, in particular, can leverage multiple sequences optimised to differentiate various tissue features to provide a more comprehensive assessment of tumour structure. In the first part of this Chapter two semantic MRI features of resected CRLM are assessed, the proportion of viable tumour and the presence of a fibrous capsule. These histological features, discussed in detail in Chapter 4, may be amenable to visual assessment and could be clinically actionable. In collaboration with colleagues from IBME, I then used a semi-automated methodology based on DCE-MRI to determine the proportions of viable tumour and compare these with histologically defined subregions.

Functional imaging techniques provide quantitative and semi-quantitative outputs which can describe different aspects of tumour biology *in vivo*. In the second part of this Chapter, two methods for assessing tumour perfusion, pCT and DCE-MRI, are compared with IHC assessment of tumour microvasculature, hypoxia and viability. The subregion segmentation developed above is applied to the PK parameter maps derived from DCE-MRI. Finally, the SUV parameters derived from ^{18}F -FDG-PET are compared to the pCT and DCE-MRI parameters and IHC.

2. Chapter Methods

i. Visual imaging assessment

The assessment was limited to 19 metastases in 15 patients who did not receive neoadjuvant chemotherapy and had pre-operative MRI within 56 days (8 weeks) of liver metastasectomy. Metastases less than 10 mm were excluded.

The visual assessment was performed by a Radiologist with approximately 30 years experience reporting liver MRI, who was blinded to the results of the histopathology analysis. The features were assessed using the pre- and post-contrast T1 weight imaging with fat saturation (LAVA) acquisitions. A fibrous capsule was considered to be present when a lesion demonstrated progressive peripheral enhancement after IV contrast relative to background liver that was persisted into the delayed phase of imaging, 2 minutes after IV contrast administration. Peripheral rim enhancement may also be observed due to compressed peripheral hepatocytes; this typically shows earlier hyperenhancement and becomes isodense to liver in the delayed phase. The proportion of viable tumour was assessed in quartiles (0-25%, 26-50%, 51-75% and >75%) based on the proportion of tumour that was perceived to demonstrate enhancement and washout compared with the pre-contrast imaging.

ii. *Image-histopathology alignment*

As described in Methods (Section 4.i), for the patients recruited to the prospective FILM study it was possible to perform the histopathological cut-up and orientate the specimens to match the study imaging. Histopathology slides were scanned digitally (Aperio CS2 Slidescanner, Leica Biosystems, USA) and imported into OsiriX 2 DICOM Viewer (Pixmeo SARL, Bernex, Switzerland) as converted DICOM files (JPEG to DICOM v2.0). DCE-MRI studies were also imported. Histopathological images were scaled to match the tumour on MRI to allow for size reduction during formalin fixation: the entire lesion was manually measured in the longest axial plane (x-dimension) and in the longest perpendicular (y-dimension) at both imaging and histopathology. The histopathology images were then rescaled to match the x- and y-dimensions of the lesion on MRI.

Histopathological images were fused with the DCE-MRI 4D volume and manually aligned to match the lesion contour in the imaging space, based on the histopathological orientation and local features such as the liver capsule or local blood vessels.

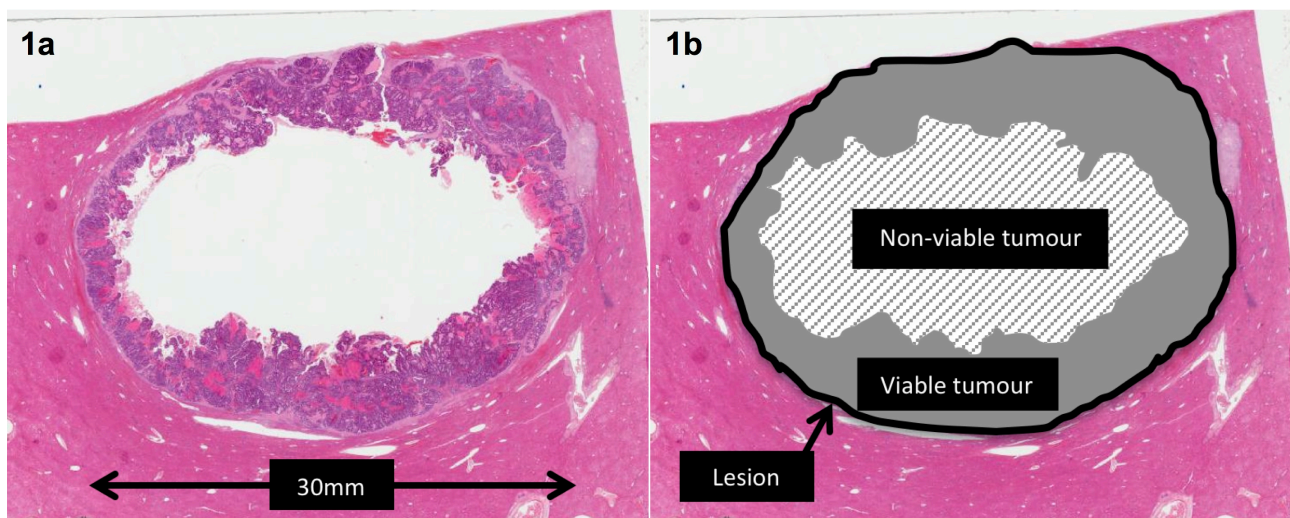
iii. Lesion subregion derivation

The entire metastatic lesion ($\text{ROI}_{\text{lesion-histopathology}}$) and the subregion(s) of viable tumour ($\text{ROI}_{\text{viable-histopathology}}$) were delineated on the histopathology image, and saved in the imaging space (Figure 5a). $\text{ROI}_{\text{viable-histopathology}}$ encompassed regions

of predominantly viable, cellular tumour. Non-contiguous tumour regions less than 500 μm in diameter were not included. To derive the non-viable tumour ROI ($\text{ROI}_{\text{nonviable-histopathology}}$), the $\text{ROI}_{\text{viable-histopathology}}$ was subtracted from $\text{ROI}_{\text{lesion-histopathology}}$.

Figure 5a: Tumour subregion delineation on H&E stained histopathology slide

Image 1a shows an H&E stained histopathology slide of a 30 mm resected colorectal liver metastasis demonstrating peripheral viable tumour with a centrally necrotic region. In image 1b the $\text{ROI}_{\text{lesion-histopathology}}$ is outlined (black line) and the regions $\text{ROI}_{\text{viable-histopathology}}$ (solid grey region) and $\text{ROI}_{\text{nonviable-histopathology}}$ (shaded grey region) are shown.



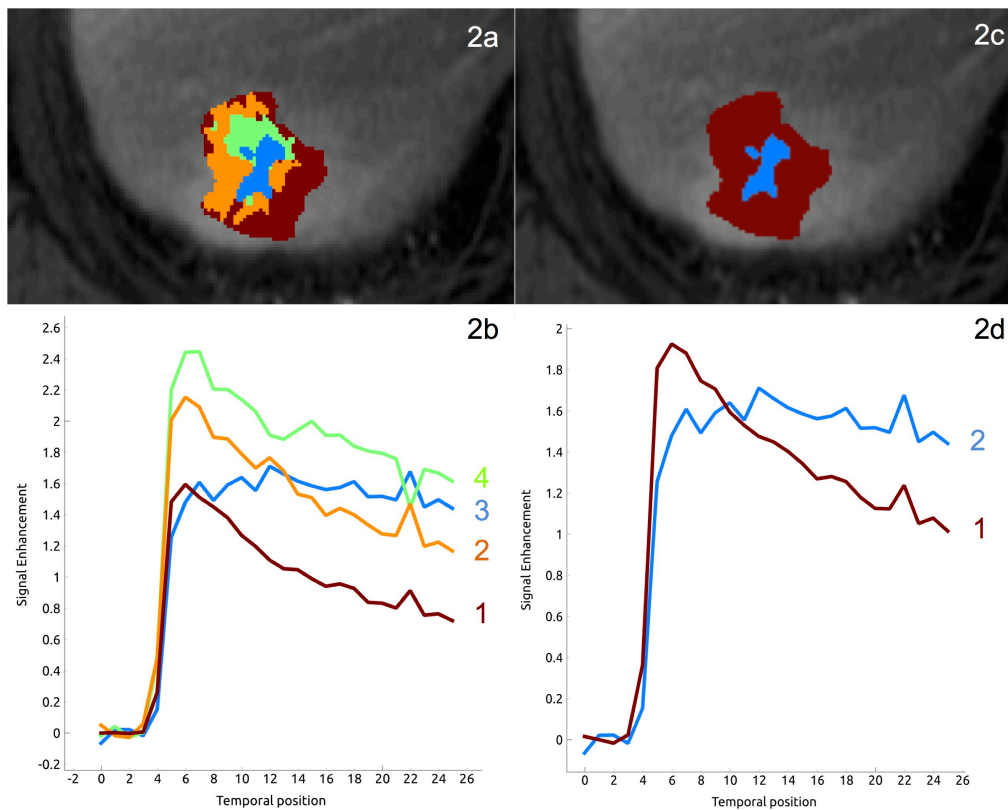
The whole volume of the metastatic lesion ($\text{VOI}_{\text{lesion-imaging}}$) was delineated on the unenhanced T1W images, or, in cases where the lesion was better defined or larger, using the T2W images, by a radiologist with seven years experience reporting liver MRI.

The imaging subregion analysis was performed using in-house software created by Dr Ben Irving at the Oxford Institute of Biomedical Engineering. To create the image-derived subregions the contrast uptake curves for the voxels within $VOI_{\text{lesion-imaging}}$ and $ROI_{\text{lesion-histopathology}}$ were analysed using a principal component analysis (PCA) of the DCE-MRI enhancement curves, followed by k-means clustering of these modes, as described previously (Irving et al., 2016). Three PCA modes were found to best separate regions of different contrast uptake characteristics, and were used to automatically define four subregions. These four subregions were subsequently manually merged into two regions: tumour, identified by a wash-in/wash-out curve, and non-tumour, where there were other contrast curves (non-enhancing or contrast accumulation; Figure 5b) as described by Kuhl et al., (1999).

Thus for the $VOI_{\text{lesion-imaging}}$ two regions were defined: $VOI_{\text{viable-imaging-subregion}}$ and $VOI_{\text{nonviable-imaging-subregion}}$. Two regions were also defined within $ROI_{\text{lesion-histopathology}}$: $ROI_{\text{viable-imaging-subregion}}$ and $ROI_{\text{nonviable-imaging-subregion}}$.

Figure 5b: Two-stage semi-automated segmentation of manually delineated liver metastasis by PCA clustering based on DCE-MRI signal-time curves

Image 2a and 2c show the four automatically defined subregions within $VOI_{\text{lesion-imaging}}$ and images 2b and 2d show the four mean signal-time curves for the equivalent regions. The three wash-in wash-out curves (Image 2b, labels 1, 2 and 4) were merged to create $VOI_{\text{viable-imaging-subregion}}$ while the remaining voxels are classified as $VOI_{\text{nonviable-imaging-subregion}}$.



iv. Functional imaging analysis

For the 14 patients included in the prospective FILM study, DCE-MRI and pCT PK parameters were derived as described in Chapter 2. For the patients in both the prospective and retrospective studies, without preoperative chemotherapy and with ^{18}F -FDG-PET within 21 days of hepatic resection, the tumour SUV was derived as described in Chapter 2.

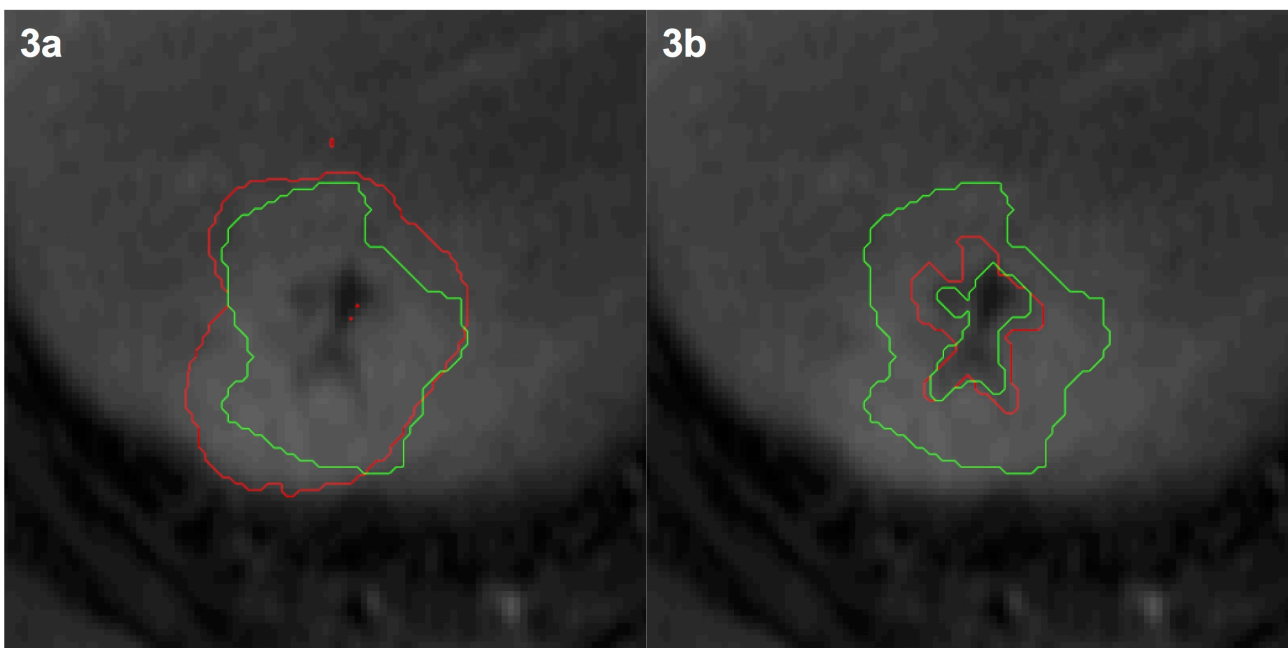
v. Data analysis

Comparison of image-derived and histopathology defined subregions

To assess the agreement between regions defined on ‘gold-standard’ histopathology with those automatically determined from imaging, the spatial overlaps of $ROI_{\text{lesion-imaging}}$ and $ROI_{\text{lesion-histopathology}}$, and $ROI_{\text{viable-imaging-subregion}}$ and $ROI_{\text{viable-histopathology}}$ were estimated using the Dice similarity coefficient (DSC) (Dice, 1945), as shown in Figure 5c).

Figure 5c: Dice similarity coefficients of between tumour subregions defined by imaging and histopathology

Image 3a shows the spatial overlap of $ROI_{\text{lesion-histopathology}}$ (green outline) and $ROI_{\text{lesion-imaging}}$ (red outline). Image 3b shows the spatial overlap of $ROI_{\text{viable-histopathology}}$ (red outline) and $ROI_{\text{viable-imaging-subregion}}$ (green outline; derived from $ROI_{\text{lesion-histopathology}}$). DSCs were 0.834 and 0.93, respectively.



Assessment of viable tumour

The percentage of viable tumour for the whole histopathology slide was reported by visual assessment by agreement of two consultant histopathologists. The percentage of viable tumour within the histopathology ROI and imaging ROI and VOI image were calculated:

- Histopathology: $100 * ROI_{\text{viable-histopathology}} / ROI_{\text{lesion-histopathology}}$
- ROI imaging $100 * ROI_{\text{viable-imaging}} / ROI_{\text{lesion-imaging}}$
- VOI imaging $100 * VOI_{\text{viable-imaging}} / VOI_{\text{lesion-imaging}}$

These values were correlated using the Pearson product-moment correlation coefficient.

PK data analysis

T1₀ and PK parameters were summarised for each of the ROI/VOIs and presented graphically. The following PK parameters comparisons were performed using paired Student's T-test. P-values <0.05 were taken to be significant.

To compare viable tumour subregions defined by imaging and defined by histopathology:

$ROI_{\text{viable-imaging-subregion}}$ and $ROI_{\text{viable-histopathology}}$ (1)

To compare viable tumour subregions defined by imaging in 2D and in 3D:

$ROI_{\text{viable-imaging-subregion}}$ and $VOI_{\text{viable-imaging-subregion}}$ (2)

To compare viable and non-viable subregions:

$ROI_{\text{viable-histopathology}}$ and $ROI_{\text{nonviable-histopathology}}$ (3)

$VOI_{\text{viable-imaging-subregion}}$ and $VOI_{\text{nonviable-imaging-subregion}}$ (4)

To compare the whole lesion with only the viable component:

$VOI_{\text{lesion-imaging}}$ and $VOI_{\text{viable-imaging-subregion}}$ (5)

Comparison of functional imaging parameters with each other and with the relevant IHC and histopathological features.

The DCE-MRI and pCT-derived functional imaging parameters, tumour SUV and the IHC percentage staining were compared using the simple linear correlation.

The functional imaging parameters of metastases with different patterns of tumour-liver interface were compared using the Student T-test.

3. Results

i. Visual imaging assessment

In the 19 assessed metastases there were 8 (42.1%, 95% CI 20.3-66.5%) encapsulated lesions and 11 non-encapsulated lesions, which is almost identical to the overall disease prevalence in the larger cohort of patients report in Chapter 3. The results are show in Table 5a below.

Table 5a: Diagnostic accuracy of MRI for the presence of encapsulation of CRLM

MRI Encapsulation	Pathological Encapsulation		Total
	Present	Absent	
Positive	6	4	10
Negative	2	7	9
Total	8	11	19

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy compared to histology were 75% (95% CI, 34.9-96.8%), 63.64 (30.8-98.1%), 60% (38.4-78.3%), 77.8% (49.3-92.7%) and 68.4% (43.5-87.4%) respectively.

There was a modest positive correlation between the proportion of viable tumour estimated from imaging and reported by histology, which did not reach statistical significance ($r = 0.419$, $p = 0.075$). The methodology of imaging assessment, dividing the groups into quartiles, may underestimate the correlation and a more

comprehensive, voxel-by-voxel assessment is discussed below. Similarly, comparing the viability of metastases scored low viability at imaging (0-50%) to those scored high viability (51-100%), there was again a non-significance difference in the viability reported by histology between the groups (22.5% vs 41.9%, $p = 0.063$).

ii. Subregion analysis

Patients

As described in the Methods section, fifteen patients were included in this prospective study and one patient was withdrawn. All of the remaining fourteen underwent hepatic metastasectomy. Of these fourteen patients, eleven patients proceeded directly to metastasectomy and three patients with synchronous metastatic disease were treated with neoadjuvant chemotherapy. DCE-MRI was performed successfully in all cases at baseline and after chemotherapy where appropriate; this provided the study dataset. Direct histological comparison with imaging was possible in nine patients: eight patients not treated with neoadjuvant chemotherapy and in one patient treated with neoadjuvant chemotherapy. The specimens of two patients (FILM012 and FILM014) were not available for analysis. One patient (FILM007) treated with chemotherapy had a complete pathological response at the time of resection. One patient (FILM001) had a delay between DCE-MRI and hepatic metastasectomy, precluding direct comparison. One patient (FILM011) had recurrence at a previous resection margin, with abnormal anatomy, and was excluded from this component of the analysis.

Histopathology analysis

Viable tumour and non-viable subregions were defined for all patients as described above (see Figure 5a).

Image analysis

Motion correction and PK analysis were performed on all cases. Imaging-derived tumour and non-tumour regions were defined for 13 of 14 lesions (Figure 5b). In one case (FILM006) the heterogeneous mix of tumour components produced wash-in wash-out tumour curves in all subregions defined at initial PCA.

Comparison of image-derived and histopathology defined subregions

The DSC for the lesions defined by imaging and histology (Figure 5c), and the viable tumour subregions defined by histology and subregion clustering are summarised in Table 5b. The lowest DSCs for the ROI_{viable} occurred in FILM006, where all tumour subregions, and therefore the entire lesion, were categorised as viable tumour, and for FILM010 and FILM015, which were small lesions with fewer than 200 pixels.

Table 5b: DSC comparing ROIs derived from imaging and histopathology

Patient	ROI _{lesion} DSC	ROI _{viable} DSC
FILM001	NA (interval chemotherapy)	
FILM002	0.923	0.784
FILM003	0.872	0.831
FILM004	0.909	0.734
FILM005	0.834	0.93
FILM006	0.746	0.635
FILM007	NA (complete pathological response)	
FILM008	0.952	0.784
FILM010	0.769	0.663
FILM011	NA (recurrent tumour)	
FILM012	NA (not assessed)	
FILM013	0.885	0.737
FILM014	NA (not assessed)	
FILM015	0.782	0.54
Mean (StDev)	0.852 (0.073)	0.738 (0.115)
Notes DSC = Disc similarity coefficient ROI _{lesion} DSC = DSC of ROI _{lesion} -histopathology with ROI _{lesion} -imaging ROI _{viable} DSC = DSC of ROI _{viable} -histopathology with ROI _{viable} -imaging		

Assessment of viable tumour

The proportion of viable tumour measured by the different methodologies is presented in Table 5c. The subregions defined by histopathology and imaging typically overestimated the proportion of viable tumour. As expected, there was a strong correlation between the proportion of viable tumour defined by subregion clustering between the ROIs and VOIs ($r = 0.934$, $p < 0.001$). Excluding patients where imaging segmentation failed (FILM006), and the lesion resected after chemotherapy (FILM010), there was a strong correlation between the proportion of viable tumour determined by visual interpretation of histopathology and by imaging ($r = 0.851$, $p < 0.01$).

Table 5c: Proportion of viable tumour by visual assessment, and calculated from the histopathology-defined and imaging-derived subregions

Patient	Visual Assessment (%)	Histopathology Subregions (%)	Imaging ROI (%)	Imaging VOI (%)
FILM001	NA (interval chemotherapy)		68	69
FILM002	15	50	27	38
FILM003	30	66	76	74
FILM004	35	58	52	47
FILM005	50	77	85	87
FILM006	30	46	100	100
FILM007	NA (complete response)		72	80
FILM008	30	51	47	44
FILM010pre	NA (interval chemotherapy)		42	46
FILM010post	5	45	59	62
FILM011	10	.	25	27
FILM012	NA (interval chemotherapy)		39	59
FILM013	30	49	34	35
FILM014	NA (not assessed)		42	48
FILM015	50	54	73	70
Mean (SD)	31.7 (11.3)	55.1 (10.5)	56.1 (22.2)	59.1 (20.8)

Notes

SD = standard deviation

PK Data Analysis

Comparisons between histopathology-defined and imaging-derived lesion subregions were performed in nine lesions from nine patients. Within-lesion comparisons of imaging-derived subregions within tumour VOIs was performed in 15 lesions in 14 patients, including one lesion both pre- and post-chemotherapy.

- (1) There were no significant differences between the K^{trans} or k_{ep} or $T1_0$ values between equivalent imaging-derived and histopathology-defined lesions or viable tumour subregions ($p>0.1$).

- (2) There were no significant differences between the K^{trans} or k_{ep} or $T1_0$ values between equivalent 2D ($ROI_{viable-imaging-subregion}$) and 3D ($VOI_{viable-imaging-subregion}$) subregions ($p>0.1$).
- (3) There were significant differences between the K^{trans} and k_{ep} values for the viable tumour and non-tumour subregions defined by histopathology ($p<0.05$): $ROI_{viable-histopathology-subregion}$ had significantly higher K^{trans} (0.48 vs 0.28 min^{-1} ; $p = 0.039$) and k_{ep} (1.10 vs 0.71 min^{-1} ; $p = 0.024$) than $ROI_{nonviable-histopathology-subregion}$. There was no significant difference in $T1_0$ values between viable tumour and non-tumour subregions ($p>0.1$).
- (4) There were significant differences between the K^{trans} , k_{ep} and $T1_0$ values for the viable tumour and non-tumour subregions defined by imaging ($p<0.05$): $VOI_{viable-imaging-subregion}$ had significantly higher K^{trans} (Figure 5d and Figure 5f; 0.41 vs 0.22 min^{-1} ; $p<0.001$) and k_{ep} (Figure 5e; 1.00 vs 0.63 min^{-1} ; $p<0.001$) and significantly lower $T1_0$ values (1.03 vs 0.89 s; $p = 0.010$) than $VOI_{nonviable-imaging-subregion}$.
- (5) There were significant differences between the K^{trans} and k_{ep} values for the whole lesion and the viable tumour subregions: $VOI_{viable-imaging-subregion}$ had significantly higher K^{trans} (Figure 5d and Figure 5f; 0.41 vs 0.33 min^{-1} ; $p<0.001$) and k_{ep} (Figure 5e; 1.00 vs 0.83 min^{-1} ; $p<0.001$) than $VOI_{nonviable-imaging-subregion}$. There was no significant difference in $T1_0$ values between viable tumour subregions and the whole lesion.

Figure 5d: Mean K^{trans} of CRLM (dark blue) and of image-derived tumour (purple) and non-tumour subregions (green)

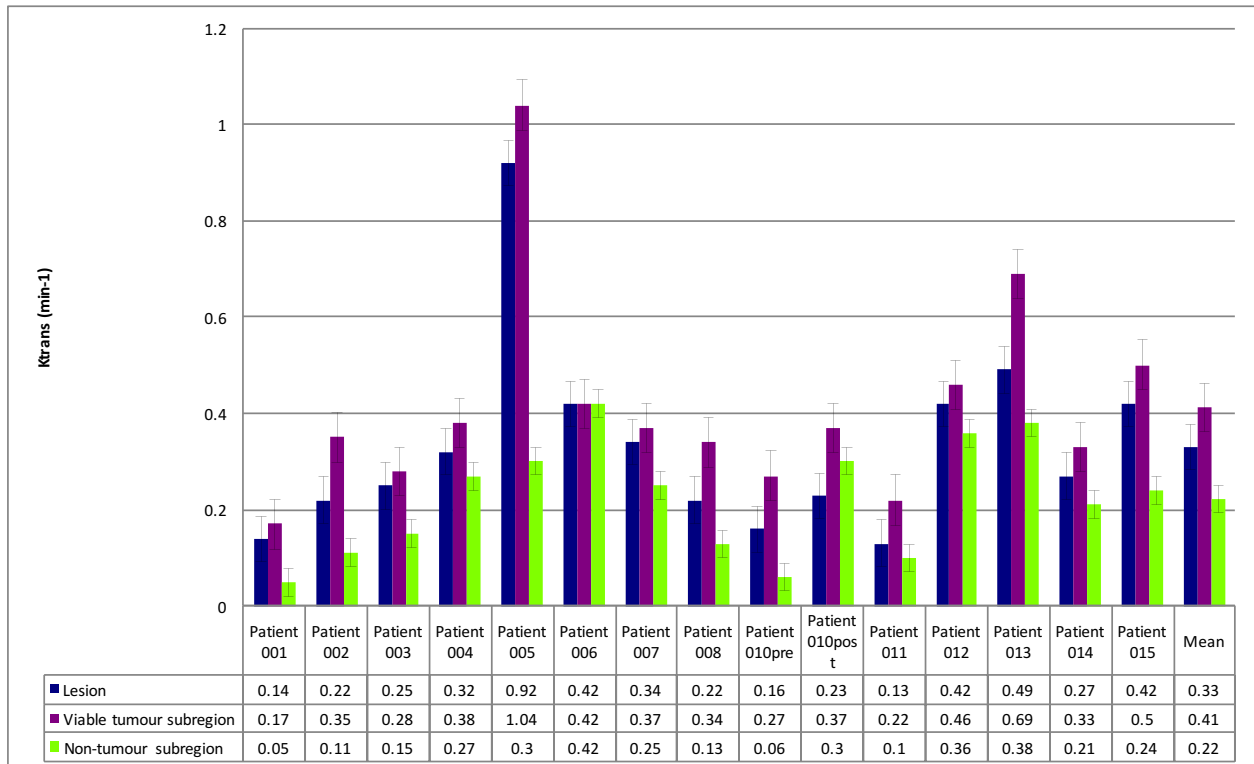


Figure 5e: Mean k_{ep} of CRLM (dark blue) and of image-derived tumour (purple) and non-tumour subregions (green)

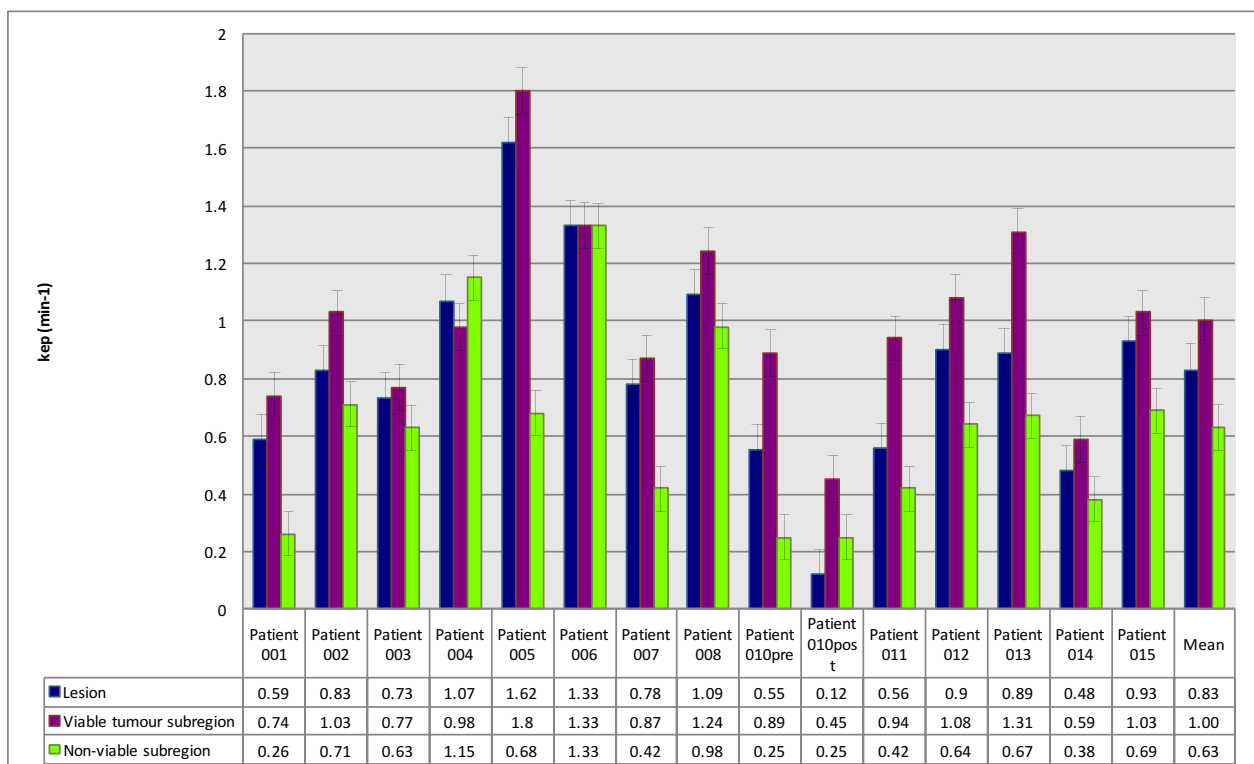
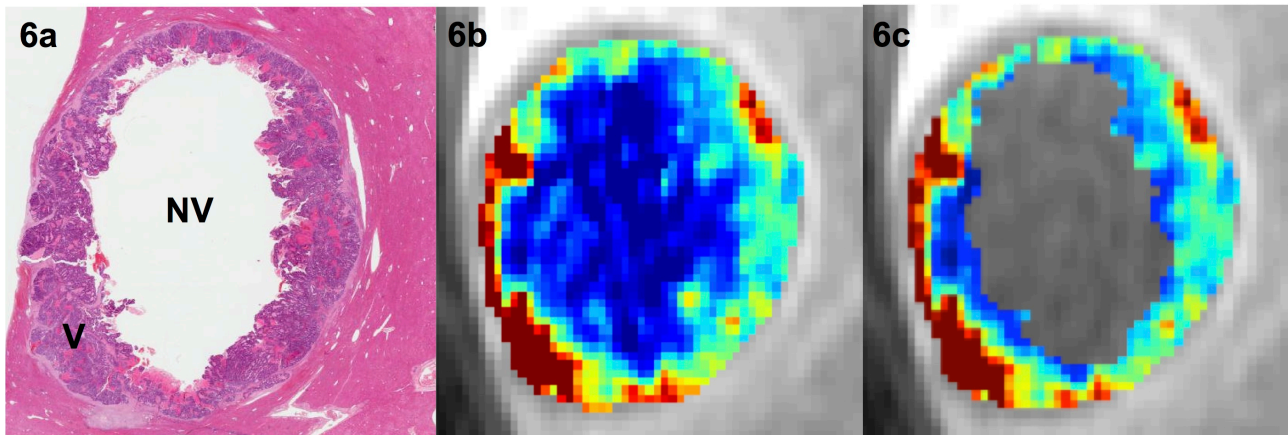


Figure 5f: Subregion analysis of K^{trans} parameter maps

Image 6a is a resected colorectal liver metastasis with central non-viable tumour (NV) and a rim of viable (V) tumour. Image 6b is the K^{trans} map for the whole lesion and Image 6c is the K^{trans} map for the viable tumour subregion only



ii. Functional imaging parameter analysis

The functional imaging parameters of the 14 patients in the prospective FILM study are shown in Table 5c below. The related IHC and histological features used in the comparisons are shown in a separate table (Table 5d) above the relevant sections.

Table 5d: FILM Study functional imaging parameters

Patient	Size	Mean K^{trans} (min^{-1})		Mean k_{ep} (min^{-1})		BV	BF	MTT	SUVmax
	Area (cm^3)	Lesion	Tumour	Lesion	Tumour	ml/ 100mg	ml/100mg/ min	(s)	
FILM001	4.08	0.14	0.17	0.59	0.74	17.30	246.00	7.51	16.3
FILM002	4.62	0.22	0.35	0.83	1.03	13.80	70.58	13.32	9.1
FILM003	2.05	0.25	0.28	0.73	0.77	4.91	37.10	9.20	6.1
FILM004	3.30	0.32	0.38	1.07	0.98	24.69	172.00	11.66	6.7
FILM005	9.55	0.92	1.04	1.62	1.80	30.74	223.63	12.37	3.5
FILM006	4.86	0.42	0.42	1.33	1.33	17.13	202.35	6.92	11.8
FILM007	8.05	0.34	0.37	0.78	0.87	16.51	74.40	14.44	10.9
FILM008	14.66	0.22	0.34	1.09	1.24	6.94	96.82	5.97	5.1
FILM010	3.93	0.16	0.27	0.55	0.89	7.08	72.20	9.62	19.8
FILM011	6.39	0.13	0.22	0.56	0.94	8.65	104.99	11.92	18.5
FILM012	2.29	0.42	0.46	0.90	1.08	17.28	69.75	16.64	16.3
FILM013	3.90	0.49	0.69	0.89	1.31	8.68	60.39	13.93	25.0
FILM014	20.10	0.27	0.33	0.48	0.59	11.78	148.96	8.40	23.7
FILM015	1.22	0.42	0.50	0.93	1.03	17.70	365.81	6.23	9.2
Average	6.36	0.34	0.42	0.88	1.04	14.51	138.93	10.58	13.0

Notes

Lesion = whole lesion, Tumour = viable tumour subregion
 BV = blood volume, BF = blood flow, MTT = mean transit time

Comparison of DCE-MRI and pCT pharmacokinetic parameters

There was a positive correlation between BV and lesion K^{trans} ($r = 0.682$, $p = 0.007$) and a weaker positive correlation between BV and tumour K^{trans} ($r = 0.585$, $p = 0.028$; Figure 5g). There was no correlation between BF or MTT and K^{trans} .

There was a positive correlation between BV and lesion k_{ep} ($r = 0.650$, $p = 0.012$) and a non-significant positive correlation between BV and tumour k_{ep} ($r = 0.481$, $p = 0.082$; Figure 5h). There was no correlation between BF or MTT and k_{ep} .

Comparison of DCE-MRI and pCT pharmacokinetic parameters with tumour SUV

Tumour PK parameters and SUV could be compared at baseline in all 14 patients. There was no correlation of tumour K^{trans} with SUV for either the whole lesion or tumour subregion (Figure 5i). There was a negative correlation between SUV and whole lesion k_{ep} ($r = -0.591$, $p = 0.026$; Figure 5j). A weaker and non-significant negative correlation was observed between SUV and tumour k_{ep} .

Figure 5g: Blood volume compared with Ktrans

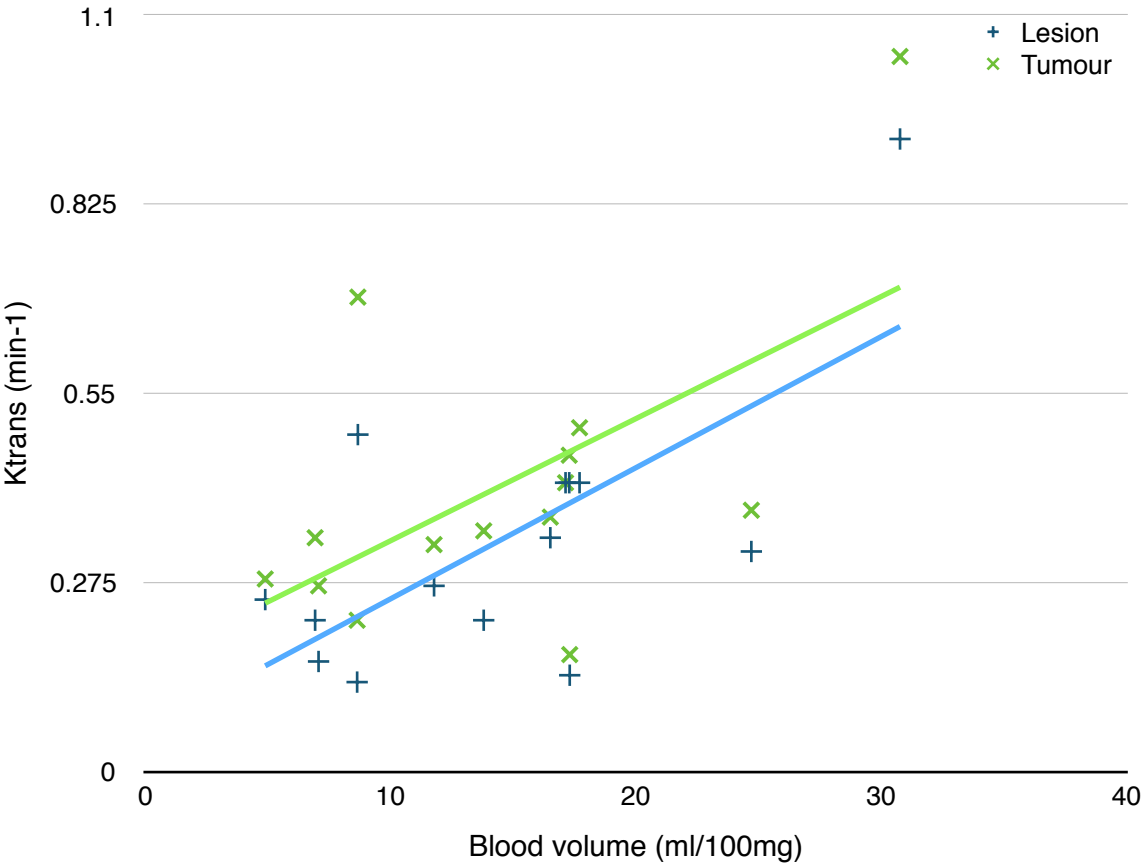


Figure 5h: Blood volume compared with kep

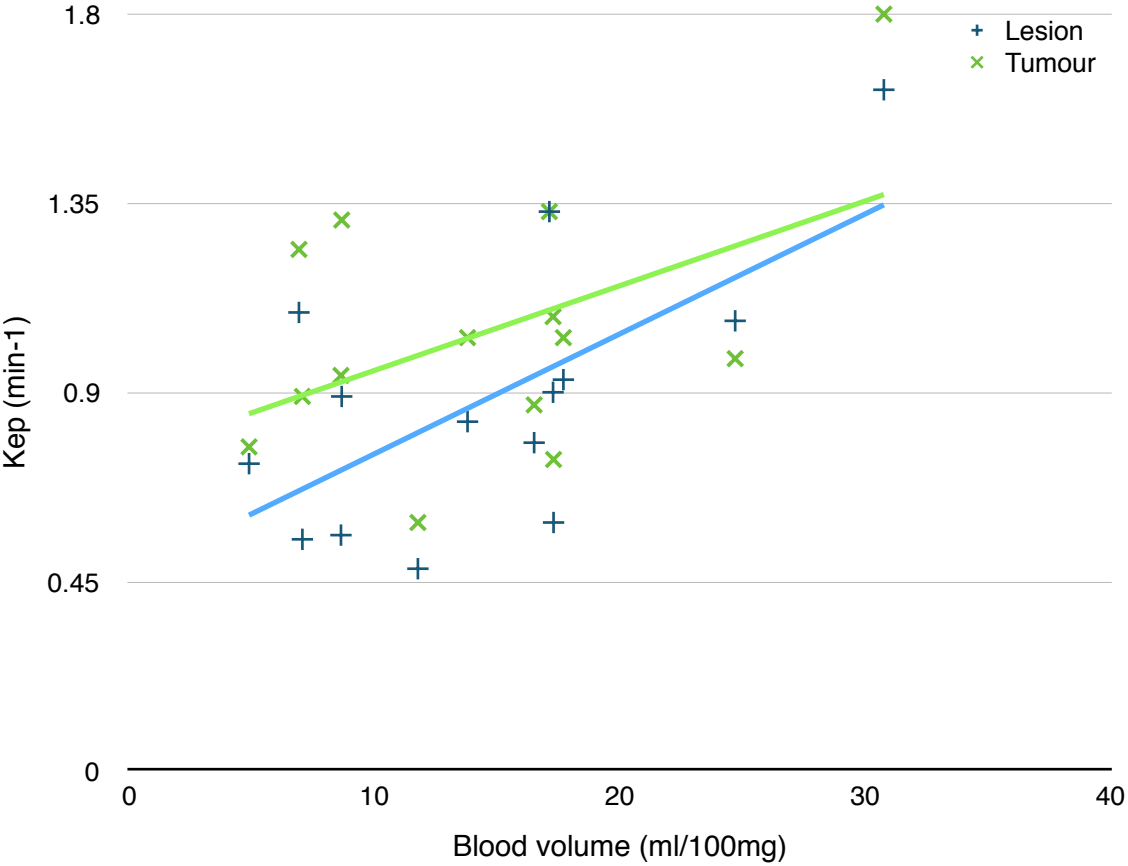


Figure 5i: SUVmax compared with Ktrans

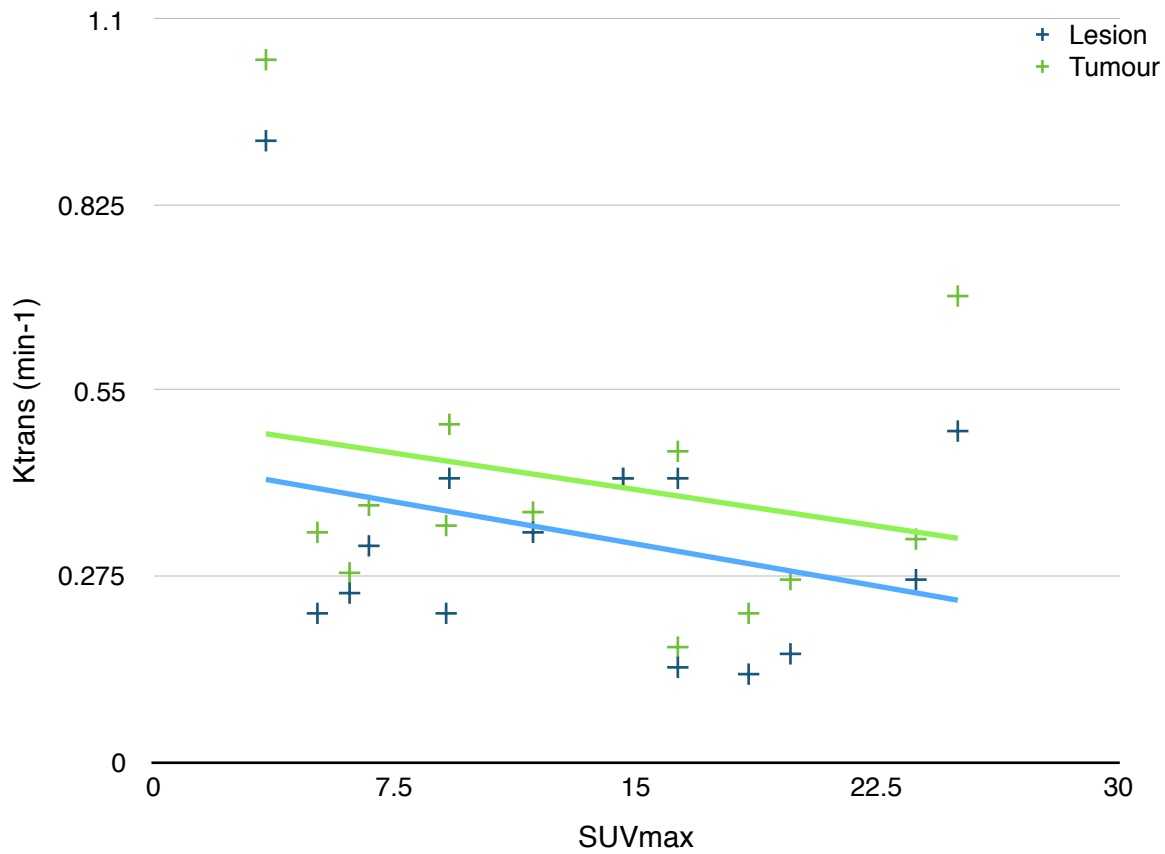


Figure 5j: SUVmax compared with Kep

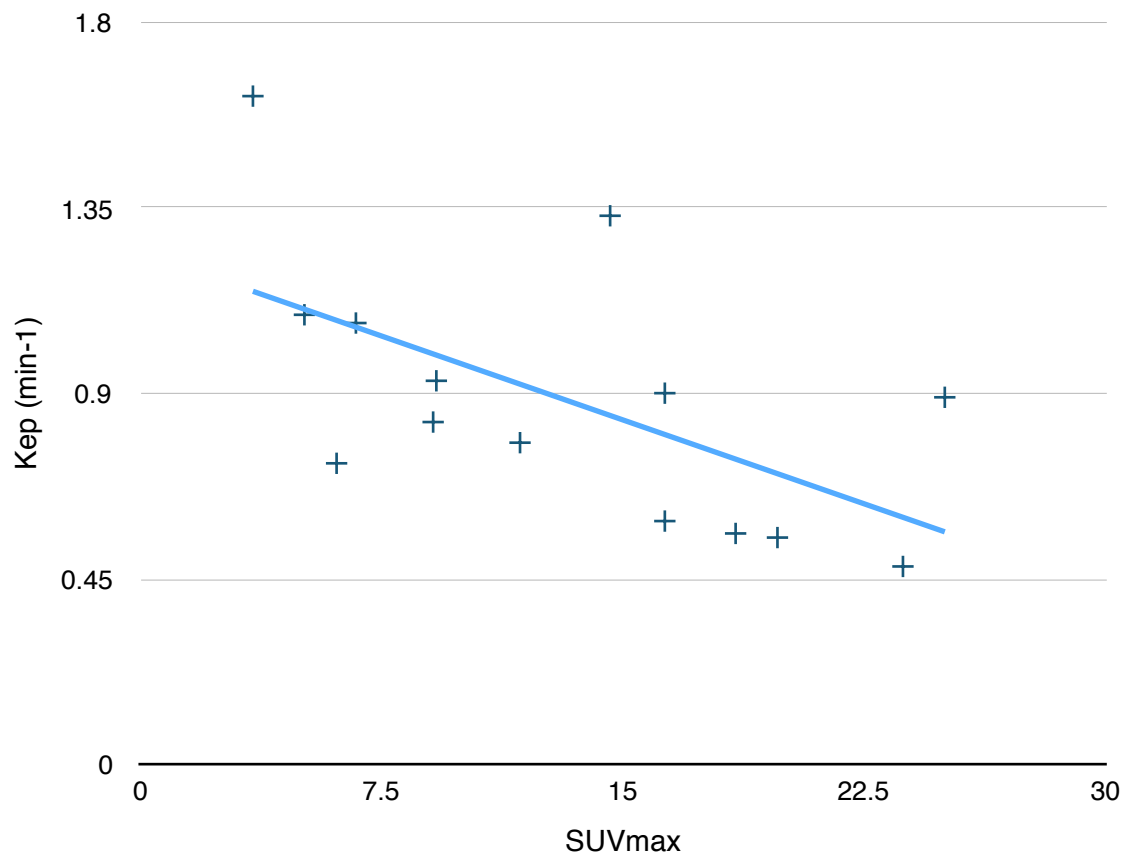


Table 5e: Prospective FILM study selected histological and IHC phenotypes

Patient	MVD				ki67		CA9		GLUT1		Vermeulen [§]	Sirwardana [@]
	1	2	3	Av	%	H/L#	%	H/L#	%	H/L#		
FILM001	5	6	5	5.3	80	1	70	1	20	0	P	NE
FILM002	3	5	5	4.3	60	0	80	1	60	1	D	E
FILM003	3	2	3	2.7	80	1	100	1	40	1	R	NE
FILM004	5	5	5	5.0	80	1	60	1	40	1	D	E
FILM005	5	6	6	5.7	60	0	40	1	10	0	P	NE
FILM006	6	5	6	5.7	90	1	30	0	40	1	D	E
FILM007												
FILM008	3	3	3	3.0	60	0	10	0	50	1	D	E
FILM010	4	3	4	3.7	10	0	90	1	5	0	D	E
FILM011	3	5	3	3.7	20	0	5	0	60	1	D	E
FILM012												
FILM013	4	5	6	5.0	70	1	20	0	50	1	P	NE
FILM014												
FILM015	5	4	5	4.7	90	1	40	1	60	1	D	E

Notes

MVD = microvessel density, CA9 = carbonic anhydrase 9, GLUT1 = glucose transporter 1.

#H = high expression, L = low expression

§Vermeulen Patterns: P = pushing pattern, D = desmoplastic pattern,

@Sirwardana Patterns: E = encapsulated, N = non-encapsulated

Comparison of DCE-MRI and pCT pharmacokinetic parameters with tumour vascularity, hypoxia, GLUT1 expression and viability

There was a non-significant negative correlation between lesion k_{ep} and CA9 ($r = -0.3384$, $p = 0.308$; Figure 5k) and between tumour k_{ep} and CA9 ($r = -0.5109$, $p = 0.109$). There was no correlation with BV, BF, MTT or K^{trans} with CA9, or correlation of any of the imaging parameters with ki67 or GLUT1 expression.

Figure 5k: Kep compared with CA9

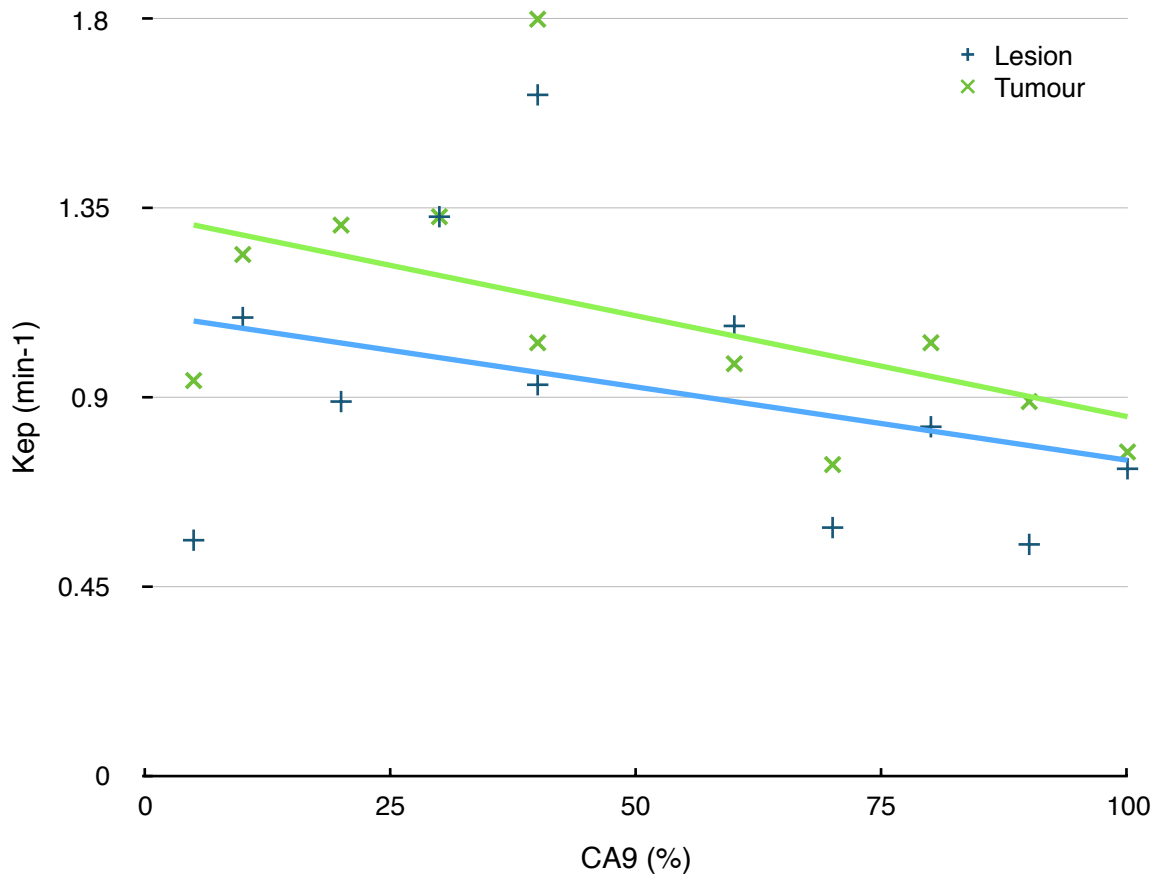


Figure 5l: Blood volume compared with microvessel density

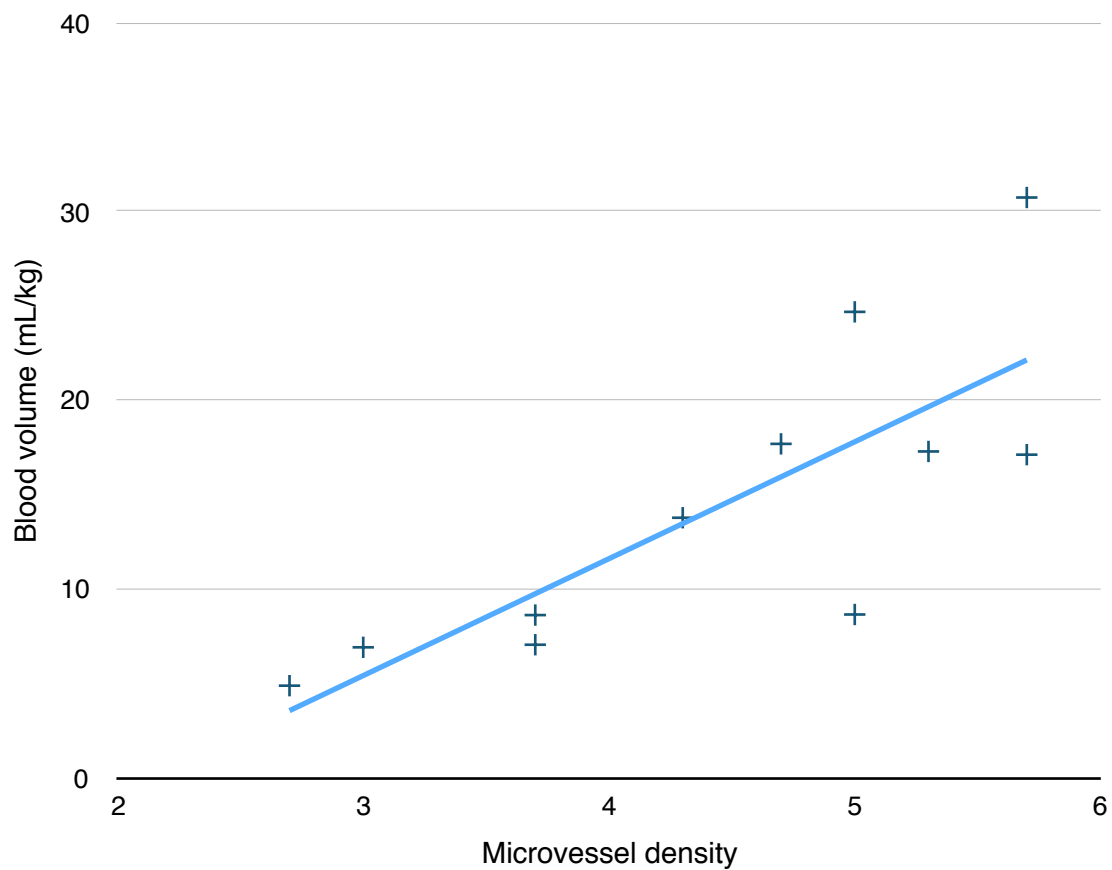


Figure 5m: Blood flow compared with microvessel density

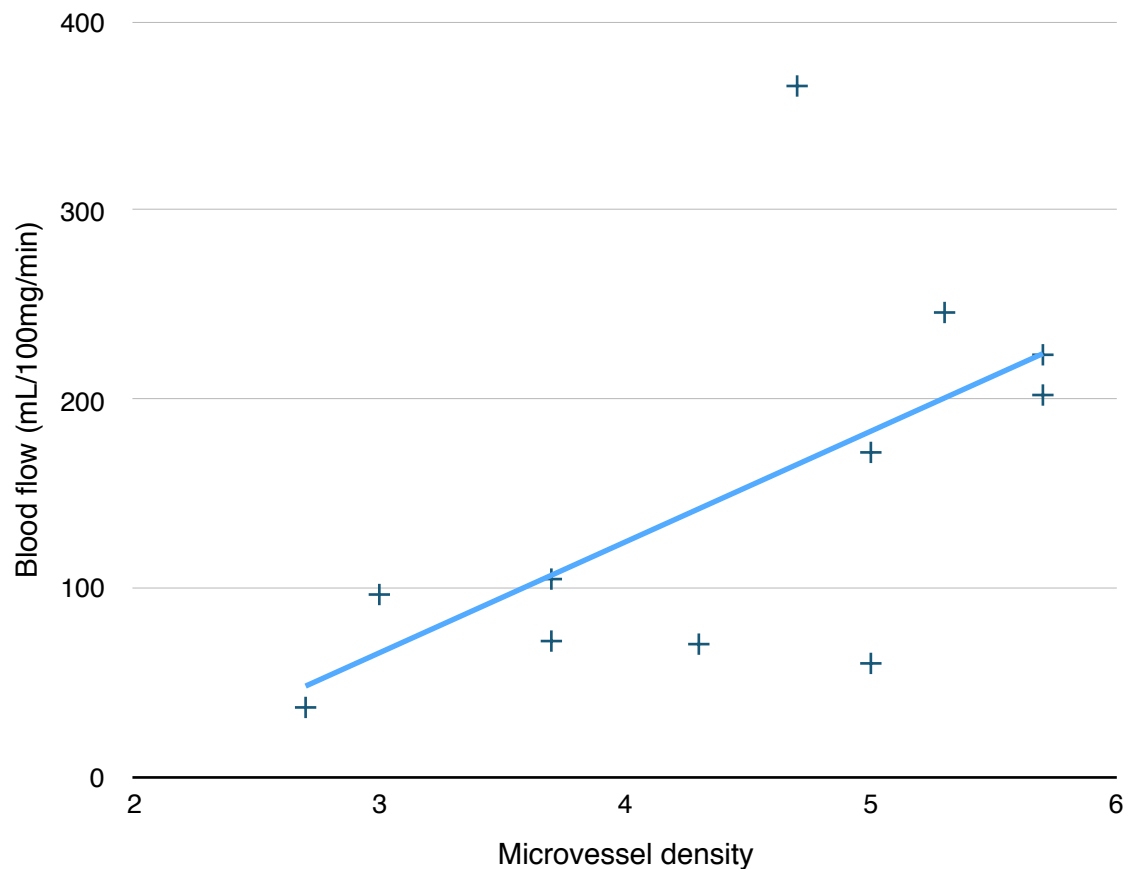
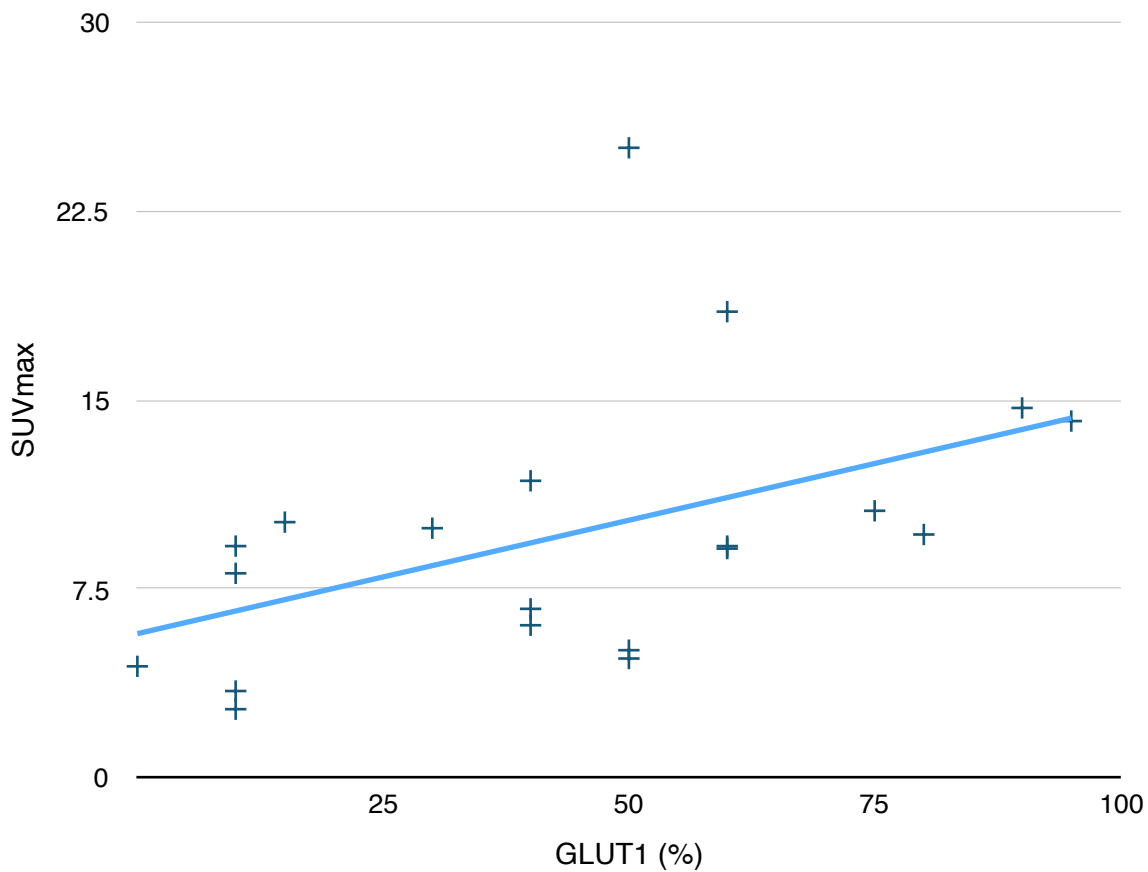


Figure 5n: SUVmax compared with GLUT1 expression



There was a significant positive correlation between BV and MVD ($r = 0.775$, $p = 0.0084$; Figure 5l) and between BF and MVD ($r = 0.603$, $p = 0.0494$; Figure 5m), and non-significant positive correlations between K^{trans} and MVD (lesion 0.582 , $p = 0.060$) and k_{ep} and MVD (lesion $r = 0.521$, $p = 0.100$).

There was no difference between the pattern of liver-tumour interface and the pCT or DCE-MRI PK parameters.

Comparison of tumour SUV with ki67, CA9, GLUT1 expression, viability and histological features.

Tumour SUVmax could be compared with IHC and histological features in 20 metastases in 16 patients. The SUVmax compared with IHC and histological features are shown in Table 5f.

There was a significant positive correlation between SUVmax and GLUT1 ($r = 0.478$, $p = 0.033$; Figure 5n) and, accordingly, low GLUT1 expressing tumours had lower SUVmax (6.86 vs 11.17), although this was non-significant ($p = 0.087$). There was no correlation between SUVmax, SUVpeak or SUVmean and ki67, CA9 or the proportion of viable cells. There was no difference in SUVmax between the different patterns of liver-tumour interface.

Table 5f: SUVmax compared with histological features and IHC

Metastasis (n = 20)	SUVmax	ki67		CA9		GLUT1		Viability	Vermeulen ^s	Sirwardana [@]
		%	H/L [#]	%	H/L [#]	%	H/L [#]			
CRLM05	9.20	80	1	50	1	10	0	10	D	E
CRLM24	9.91	70	1	40	1	30	0	30	R	NE
CRLM31-1	10.15	90	1	60	1	15	0	20	P	NE
CRLM31-2	4.74	60	0	60	1	50	1	50	P	NE
CRLM38-1	8.12	60	0	30	0	10	0	30	P	NE
CRLM38-2	4.43	90	1	0	0	0	0	50	P	NE
CRLM41-1	14.16	70	1	80	1	95	1	40	D	E
CRLM41-2	14.68	50	0	100	1	90	1	20	D	E
CRLM47	9.66	50	0	30	1	80	1	50	P	NE
CRLM50	10.60	90	1	10	0	75	1	60	R	NE
FILM002	9.10	60	0	80	1	60	1	15	D	E
FILM003	6.06	80	1	100	1	40	1	30	R	NE
FILM004	6.71	80	1	60	1	40	1	35	D	E
FILM005	3.45	60	0	40	1	10	0	50	P	NE
FILM006	11.79	90	1	30	0	40	1	30	D	E
FILM008	5.07	60	0	10	0	50	1	30	D	E
FILM008	2.73	30	0	5	0	10	0			
FILM011	18.50	20	0	5	0	60	1	10	D	E
FILM013	25.00	70	1	20	0	50	1	30	P	NE
FILM015	9.20	90	1	40	1	60	1	50	D	E
Median	9.20	70		40		45		30		

Notes

SUVmax = standardised uptake value (maximum)

CA9 = carbonic anhydrase 9, GLUT1 = glucose transporter 1.

[#]H = high expression, L = low expression

^sVermeulen Patterns: P = pushing pattern, D = desmoplastic pattern,

[@]Sirwardana Patterns: E = encapsulated, N = non-encapsulated

4. Discussion

i. Visual imaging assessment

This was preliminary investigation of visual morphological assessment of two features of CRM, the proportion of viable tumour and the presence of a capsule. There was only a modest agreement of the proportion of viable tumour assessed by visual assessment of the imaging and histopathology. This assessment is difficult due to the varied baseline T1 signal of CRLM, and the presence of enhancement in both viable tumour and acellular components such as a fibrous tissue. To appreciate how subregions of tumour behave after IV contrast administration also requires assessment of multiple acquisitions at different phases of contrast, which is more challenging than single time-point assessments. For this reason it may be preferable to use automated or semi-automated software to analyse the post-contrast imaging and the potential utility of this methodology is explored in the second part of this Chapter.

As discussed previously the presence of encapsulation may have an impact on treatment selection and prognostication; defining this feature *in vivo* may improve patient management. We found that visual assessment has a moderately good sensitivity (75%) and NPV (77.8%) for the presence of a capsule, but only modest specificity (63.6%) and PPV (60%). This is likely to be due to the difficulty in differentiating altered enhancement due to hepatocyte compression from a true capsule, for example FILM013 had a thick surrounding rim of compressed

hepatocytes, and was a false positive for encapsulation. Some of the tumour features that are readily identifiable at microscopy are significantly smaller than the achievable in-plane resolution of clinical MRI techniques. For example, the capsular thickness in this study varied from approximately 50 μm to 2000 μm , while the in-plane resolution of MRI is typically in the region of 1 mm^3 . This does not include the loss of spatial resolution due to liver movement due to respiratory and cardiac motion during image acquisition, which occur despite routine respiratory gating for multiple breath-hold techniques. Clearly, the potential to increase scan durations for single breath-hold techniques is limited by patient tolerances. Currently, imaging of the liver is optimised for lesion detection. In the future, modified MRI sequences might allow small field of view imaging of individual lesions to facilitate morphological assessment, as has become standard practice for rectal cancer assessment (e.g. Brown et al., 1999).

ii. Subregion study

This study has used a semi-automated clustering approach based on PCA of DCE-MRI contrast uptake curves to define regions of viable tumour within CRLM. We have demonstrated that distinct subregions can be derived in the majority of patients, and that there is concordance of these subregions with carefully sectioned and coregistered histologically defined subregions.

Pre-defining a region of viable tumour can be used to direct analysis of PK parameter maps. We have demonstrated significant differences between the

mean PK parameter values of subregions of viable tumour and non-viable subregions, irrespective of whether these subregions are defined by histopathology or imaging. It is conventional to analyse PK maps by generating and interpreting data for the whole lesion: either deriving a mean contrast-uptake curve, or generating data for each voxel and pooling these data to generate summary statistics. (O'Connor et al., 2007) Importantly, we have demonstrated that the mean PK data for viable tumour produces significantly different results from the mean PK data for whole lesion analysis as is the current standard practice.

Defining a subset of voxels that represent viable tumour has two advantages. Firstly, the voxels represent biologically active tumour and parameters derived from these voxels may correlate more consistently with aspects of tumour biology and outcome. Using DCE-MRI to assess tumour phenotypes has produced mixed results. (O'Connor et al., 2007) As illustrated in our study, non-viable regions, vary significantly from lesion-to-lesion in the proportion of tumour that they represent. If data from both viable and non-viable subregions are incorporated into the summary statistics, these may not consistently reflect properties of the viable tumour. There are some approaches in the literature that have used different methodologies to analyse subsets of PK data, for example based on hot spots (Jackson et al., 2007) or using other data-driven subsets of the parameter maps. (Hayes, Padhani and Leach, 2002) Our a priori technique defines regions considered to be tumour and then derives PK data for these regions. An

advantage of this method is that the extraction of enhancement characteristics and clustering are performed separately on each case based on the variation within the lesion and, therefore, does not require measurement or estimation of the AIF, or correction for $T1_0$, to perform the segmentation. Other researchers have adopted comparable approaches, for example by defining tumour voxels by the presence of enhancement, (de Lussanet et al., 2005) or excluding pixels with PK parameter values of zero. (O'Connor et al., 2011) However, enhancement is a feature of both viable tumour and other tissues, such as fibrosis, which is a major component of many tumours, including CRLM. (Maru et al., 2010) We have taken into account the shape of the contrast-uptake curve in defining viable tumour and used a spatially-matched histological gold standard, to confirm voxel-wise concordance of our segmentation methodology.

Secondly, our subregion representation could also be used to quantify viable tumour, which is prognostically relevant (Maru et al., 2010), as is the regression of viable tumour in response to chemotherapy (Brouquet et al., 2013). Currently, formal response assessment for CRLM is made using by RECIST 1.1 criteria (Eisenhauer et al., 2009) based on changes in lesion size alone. These do not include any morphological criteria, although it is recognised that morphological changes may improve response characterization. (Choi et al., 2007) In a small number of patients, our imaging-derived estimates demonstrated a strong correlation with the histopathological assessment of the proportion of viable tumour, for untreated metastases. We did not have a large enough cohort of

metastases resected after chemotherapy to assess whether the technique could be useful post-treatment. The ROI-based techniques tended to overestimate the amount of viable tumour compared with visual histological assessment. This is likely to be because the resolution of imaging is lower than microscopic visual assessment, and is insufficient to resolve admixed viable and non-viable tumour components. This was similar to the methodology described by Monsky et al. (2016) for soft tissue sarcomas, which demonstrated overall correlation, but did not include spatially-matched tumour regions. Our technique could be adapted to generate a composite metric of the volume of viable tumour cells, which may more accurately reflect disease burden and response. Although we have developed this in the setting of resectable liver metastases, in order to allow direct histopathological correlation, this approach could be applied to the larger population of patients with metastatic liver disease who might be treated with palliative chemotherapy, SIRT or SBRT .

One of the limitations of our study is its small sample size. While we were able to show significant differences in PK parameters between tumour subregions, we had insufficient number of patients to assess whether imaging estimates of viable tumour could be used both before and after treatment. This requires further investigation using a larger dataset. One additional challenge of the small cohort and large number of comparisons performed is potential for false-positive significant associations and reduced power to detect associations (Type I and Type II statistical errors, respectively). In these experimental analyses, a

conventional p-value threshold of 0.05, was used, without corrections for multiple comparisons which would have been required in a clinical trial. To mitigate this limitation and allow readers to draw their own conclusions on the significance of the reported results, all p-values between 0.001 and 0.1 and stated in full.

Additionally, the pathological assessments were in two dimensions, as sections were only taken at the central level, rendering it impossible to reconstruct the entire three-dimensional structure of the liver metastases. As a result, the imaging-histopathological correlation analysis could only be performed in 2D. There were also several manual steps in our methodology. Firstly, despite careful sectioning, the imaging-histopathology registration involved a manual alignment step to account for fixation and orientation. Secondly, the histopathological subregions were manually defined by experienced observers, rather than automatically defined. Finally, our segmentation strategy involved a supervised stage where automatically generated subregions were merged to create tumour and non-tumour regions. We recognise that a fully automated segmentation would be preferred.

In summary, tumour segmentation based on DCE-MRI characteristics can define regions of viable tumour in CRLM. This may have applications for quantitative imaging analysis, and clinical utility as a method of more accurately quantifying tumour burden.

iii. Functional imaging parameter analysis

The PK parameters K^{trans} and k_{ep} derived from DCE-MRI were shown to correlate positively with the pCT-derived blood volume. The correlation of K^{trans} and k_{ep} values derived from the tumour subregion was weaker than those seen when comparing the whole lesion values, and this is likely to be because the pCT-derived parameters were not subject to subregion analysis and therefore described mean values for the whole lesion. There is very little comparative data on pCT and DCE-MRI performed in the same patients. A study in 17 patients with head and neck cancer (Bisdas et al., 2007) demonstrated a correlation between BF derived from DCE-MRI and pCT, although here the DCE-MRI technique was designed to measure first pass perfusion. In 19 patients with rectal cancer, Kierkels et al., (2010) demonstrated that K^{trans} values derived from pCT and DCE-MRI correlated positively, although in this case the pCT acquisition was extended to 100 seconds to allow assessment of steady state. In a small study of patients with rectal cancer receiving a novel radiosensitising agent, nelfinavir, Hill et al. (2016) found that there were parallel increases in BF and K^{trans} in patients undergoing radiotherapy. The data presented here is the first to show a positive correlation of K^{trans} and k_{ep} with BV. A correlation between K^{trans} and BV is expected since BV is dependent on blood flow, the size of the vascular bed and, in the setting of increased vascular permeability that exists in tumours, vascular leakiness. K^{trans} also depends on the vascular permeability and concentration gradient between the vascular bed and the extracellular extravascular space established by tumour perfusion. It is useful to understand the correlation that

exists between these parameters, which measure interrelated components of tumour perfusion. In both clinical and research practice it may be more practical to use one technique or another, without necessarily needing to perform both techniques.

There was a significant positive correlation between BV and BF and MVD assessed by CD34 staining. Increased expression of CD31, CD34 and CD105 is reported in many tumour types due to microvascular neoangiogenesis, and has been linked to poorer outcomes in CRC (Des Guetz et al., 2006). Numerous studies have found positive correlations between MVD and DCE-MRI parameters, across numerous tumour types including breast cancer (Jia et al., 2014), glioma (Jia et al., 2015), GISTs (Consolino et al., 2016) and CRC (Yeo et al., 2015). Although MVD should theoretically correlate strongly with pCT parameters, particularly BV as it is a reflection of the size of the vascular bed, the relationship has been variable in the literature. For example, no consistent relationship between pCT parameters and MVD was found by two groups studying CRC (Feng et al., 2009, Dighe et al., 2013) whereas another group found a positive correlation between BV and permeability surface area product and MVD (Goh et al., 2008) and similar relationships have been found in other tumour types (Chen et al., 2010, Osimani et al., 2012, Spira et al., 2013).

None of the markers of proliferation or hypoxia were significantly correlated with the functional imaging parameters, although there was a non-significant negative

correlation between k_{ep} and the hypoxia marker CA9. There was also a negative correlation between k_{ep} and SUVmax. As discussed below, SUVmax may be a surrogate marker of hypoxia. A negative relationship between perfusion parameters and markers of hypoxia might be expected, since poorly perfused tumours may be more hypoxic. This anticipated relationship has been demonstrated in imaging studies in head and neck cancer (Donaldson et al., 2011) although the opposite relationship was found in another study of head and neck cancer (Newbold et al., 2009). This inconsistency, and the failure to demonstrate significant correlations in the current study cohort, may be due to the complex relationship between tumour perfusion, angiogenesis and hypoxia. Neoangiogenesis, and potentially improved perfusion, may be driven by hypoxia in some circumstances, whereas in other tumour microenvironments hypoxia arise due to failure of tumour angiogenesis to develop sufficient perfusion.

The various patterns of liver-tumour interface were not correlated with differences in pCT or DCE-MRI parameters. This comparison was hampered by the relatively small numbers in each group, in particular the replacement group ($n=1$). It has been reported that this group in particular may have a different mechanism for developing tumour vascularity, co-opting normal vessels within the liver, rather than promoting conventional neoangiogenesis (Frentzas et al., 2016). Another issue with the classification systems is that the desmoplastic and encapsulated groups incorporate tumours both with more widespread desmoplasia and more conventional pushing tumours which have developed a fibrotic capsule. In terms

of their perfusion characteristics it may be that the latter group have more in common with the non-encapsulated pushing group. Unfortunately, there were too few metastases in each of these smaller subgroups to make meaningful comparisons. While it may be possible to use non-invasive perfusion imaging techniques to differentiate non-angiogenic from angiogenic tumours, and this may be desirable for treatment selection (Donnem et al., 2013; Frentzas et al., 2016), it has not been possible to test this hypothesis in this small cohort and case mix.

There was a moderately significant positive correlation between tumour SUVmax and GLUT1 expression. GLUT1 is the most prevalent glucose transporter and increased expression is reported in many solid organ tumours compared with normal tissue. It is thought to be an important mechanism for delivering the increased glucose demands of ATP generation through aerobic glycolysis frequently present in tumours, including CRC. FDG is a glucose analogue and the function of GLUT1 is to allow glucose uptake, therefore a positive correlation is expected and has been reported across tumour types, including CRC (Gu et al., 2006, Riedl et al., 2007). However, although these correlative studies suggest the dominant mechanism of glucose uptake in CRC is via GLUT1, the mechanisms of glucose transport are complex. There are fourteen GLUT transporters in the the facilitative glucose transporter family, as well as the family of six sodium-dependent glucose transporters (SGLT) (Szablewski 2012), all of which may contribute to glucose uptake in tumour. GLUT3, for example is also over-

expressed in many tumours, although inconsistently in CRC and one study reported no correlation between GLUT3 expression and SUVmax (Riedl et al., 2007). However, the complexity of glucose transporter mechanisms in cancer, as well as other uncontrolled factors that might influence FDG-uptake, such as the effect of HIF-1a expression (Iwamoto et al., 2014) or microvascular permeability, or measurement of SUVmax, such as tumour size, is likely to explain the moderate rather than strong correlation between SUVmax and GLUT1 expression.

There was no correlation between proportion of cellular viability, CA9 or ki67 expression and FDG uptake. Cellular viability might be expected to correlate with PET-derived parameters as only viable cells will utilise glucose and it is used as a response marker in many tumours. SUVmax is a measure only of the maximum region of uptake, whereas the proportion of viable tumour was assessed across the entire metastasis. However, even PET-derived parameters that summarise FDG-uptake over larger regions (SUVpeak) or across the whole tumour (SUVmean) did not show any consistent relationship. The proliferative marker ki67 expression has been correlated with SUVmax in CRC (Riedl et al., 2007) and other tumours (Han et al., 2009; Kajary et al., 2015). Similarly CA9 did not correlate with SUVmax. CA9 expression might be indirectly linked to FDG-uptake since both are induced in the presence of hypoxia. However, since many colorectal tumours use glycolysis even in the absence of hypoxia (aerobic glycolysis), glucose utilisation may not be consistently linked to hypoxia. It is uncertain why these relationships were not found in this study, possibly due to the small number of patients studied

and a less direct relationship between these markers and FDG-uptake in comparison with GLUT1 expression.

A limitation of the work presented in this Chapter is the relatively small number of patients recruited to the prospective study in whom the perfusion imaging was performed. This limited the potential to investigate subgroups, particularly the different patterns of liver-tumour interface. In general, the proportion of patients with liver metastases from CRC who go on to have liver metastasectomy is a small subset of the patients with metastatic CRC. Studying this subset has the advantage of allowing careful histopathological correlation with imaging which allowed detailed subregion analysis, but limited recruitment. Future studies might pragmatically investigate both oligometastatic and polymetastatic populations, using a combination of biopsy and surgical pathology to widen the study population.

In conclusion, this work has analysed morphological and functional perfusion imaging parameters derived from MRI, pCT and DCE-MRI and demonstrated relationships between these parameters and prognostically and therapeutically relevant tumour histological and immunohistochemical features. A novel subsegmentation technique based on DCE-MRI has been developed in collaboration with colleagues in the Department of Engineering. These subregions correlate with whole mount histology, and there are potentially important differences in PK parameters between subregions.

Chapter 6

Discussion

Between a third and a half of patients with CRC will develop metastases during their disease course. The opportunities to treat patients who develop liver metastases with radical intent is evolving rapidly. Developments in surgical technique (laparoscopic surgery, pre-operative portal vein ligation/embolisation and two-stage hepatectomy) and local ablative therapies such as percutaneous ablation (radiofrequency and microwave ablation) and stereotactic radiotherapy (SABR/SBRT) are expanding what is technically treatable (Vibert, Canedo and Adam, 2005). Systemic chemotherapy or liver-directed SIRT can sometimes be used bridging strategies to render unresectable patients resectable (Adam et al., 2009, Chua et al., 2010). Similarly, MDTs are increasingly enthusiastic about

offering curative-intent treatment in the face of extrahepatic disease (Carpiza and D'Angelica, 2009), and this is certainly not a contraindication to hepatic metastasectomy (Adams et al., 2013)

Without a clear biological definition of oligometastases, as originally suggested (Hellman and Weischelbaum, 1995), it is arguably the therapeutic opportunity to treat that is defining our approach to patients, not just in CRC, but in a wide spectrum of other primary tumours such as breast, NSCLC and prostate cancer (Franklin et al., 2016). As the opportunity to treat oligometastases expands, the returns become smaller. The five-year survival is in the region of 40% following CRLM (Kanas et al., 2012), but this drops to between 15—26% if extrahepatic disease is resected, depending on the site (Hadden et al., 2016). As patients accumulate risk factors for recurrence, the incremental advantage of radical treatment is likely to be reduced. A major problem for this field is the paucity of randomised evidence which would provide a reference point for against which evolutions in practice could be tested, and also a context in which biomarkers could be applied and novel biomarkers tested. Clinical and imaging biomarkers have been described and have been incorporated into clinical practice, but are insufficiently robust and have not been developed alongside therapeutic trials, so can only be loosely applied, limiting their clinical utility.

The overall scope of this work was to improve the understanding of how imaging biomarkers could be developed for oligometastatic CRLM. Firstly, in Chapter 3,

existing clinical biomarkers and both previously described and novel imaging biomarkers were analysed as prognostic biomarkers in patients with resected CRLM. The number and intra- and extrahepatic distribution of metastases were significant determinants of disease-free survival, as previously described, in numerous previous series (Kanas et al., 2012). Disease staging is, and is likely to remain, the most important function of diagnostic imaging techniques, since it provides unique spatial information and therefore critical information needed for treatment planning. The intrahepatic per-lesion sensitivity of imaging, particularly MRI and PET, will continue to improve. MRI techniques now routinely incorporate DWI and liver-specific contrast agents which provide the highest sensitivity for detection the liver of metastases (Vilgrain et al., 2016). ¹⁸F-FDG-PET/CT changes management in around a quarter of patients, compared with ceCT and MRI, principally due to the detection of EHD. Development in PET technology, such as novel reconstruction methodologies, can improve signal-to-background in the liver, and so may improve per-lesion sensitivity. One important challenge of improving sensitivity for liver metastases and EHD is the phenomenon of stage-migration (Feinstein, Sosin and Wells, 1985), where more patients are determined to have poor prognosis disease based on criteria established using less sensitive techniques. This may falsely reassure clinicians of improving outcomes, which may be spurious. It will be important to re-evaluate imaging biomarkers on patients in whom the current standard-of-care imaging is available to ensure these are fit for purpose.

An investigation of ^{18}F -FDG-PET biomarkers indicated an association between higher tumour SUV and poorer outcomes. This would be expected given the results of prior studies and the observations that tumour SUV is generally positively correlated with tumour GLUT1 expression, and higher GLUT1 expression is associated with poorer outcomes in CRC (Yang et al., 2017). This adds to the growing literature on ^{18}F -FDG-PET as a biomarker in oligometastatic CRC. However, the studies in which ^{18}F -FDG-PET biomarkers have been assessed have used heterogeneous techniques and have studied between 18 and 152 patients. In contrast, the established clinical biomarkers have been tested in thousands of patients, typically with cohorts of one hundred to one thousand patients. For imaging biomarkers to contribute to multifactorial prognostic modelling, they should be equally robustly developed and validated. A major issue in general for imaging biomarker studies, when compared with tissue, or even genomic data, has been the capacity to perform retrospective analyses of large cohort, due to developments in imaging technique and variations in utilisation in clinical practice. This is particularly true of research imaging techniques, such as DCE-MRI and pCT considered elsewhere in this thesis but can also be true of routine clinical techniques. For example, the data on ^{18}F -FDG-PET biomarkers for CRLM, suggests that metabolic response to neoadjuvant therapy, whether that be chemotherapy (Lau et al., 2014) or SIRT (Gulec et al., 2011, Fendler et al., 2013), are more consistently markers of favourable prognosis than baseline parameters alone. Response criteria for PET are already clearly defined using EORTC (Young et al., 1999) or PERCIST (Wahl et al., 2009) criteria.

However, in the majority of UK centres, post-treatment ^{18}F -FDG-PET is not routine after neoadjuvant chemotherapy, and limiting the available cohorts of patients in whom metabolic response biomarkers can be assessed. Our extensive local experience of incorporating routine PET into oesophageal staging and re-staging after neoadjuvant chemotherapy is that it can contribute useful prognostic information to a prognostic model (Findlay et al., 2017; Findlay et al., 2017). These analyses were only possible due to prospective incorporation of this imaging into clinical practice. If candidate imaging biomarkers are going to be successfully developed, collection of the raw imaging data has to be consistently incorporated into patient pathways. This has to be planned up front, ideally in a multicentre approach, to allow consistency of techniques and timings.

The borderline-significant association between background SUV and hPFS was an interesting and novel finding in this study, but not without precedent in the literature. Textural analysis of the liver parenchyma in patients with cancer but without identifiable liver metastatic disease has found changes in liver texture (Miles et al., 2009). Similarly, in the ultrasound literature, changes in total liver blood flow, as estimate by Doppler perfusion index, are predictive of changes future intrahepatic metastatic relapse in patients without bulk metastatic disease (Leen et al., 1995). This is thought to be due to alterations in liver parenchymal blood flow. This altered flow could be part of a systemic inflammatory response due to a high intravascular tumour load. In NSCLC, increased bone marrow FDG-uptake was associated with elevated serum CRP and was a poor prognostic

marker for recurrence-free survival (Lee et al., 2017). Other markers of systemic inflammation: serum albumin, white cell count (WCC), neutrophil to lymphocyte ratio (NLR) and platelet to lymphocyte ratio (PLR) have all been associated with adverse outcomes in NSCLC (Imai et al., 2015, Tolia et al., 2015, Wu et al., 2015, Lee et al., 2017). Inflammation is an important promoter of metastatic invasion and is involved in preparation of the metastatic niche (Peinado et al., 2016). An alternative explanation for altered liver SUV which would explain an association with intrahepatic recurrence would be the presence of micrometastatic disease already established in the liver which is too small to be detectable by current imaging techniques. The rate of early relapse in patients undergoing hepatic resection for CRLM remains high, and the majority of patients who do have recurrence relapse within 12 months (Gomez et al., 2010). The cohort studied here had a similarly high proportion of early relapse. Furthermore, pre-operative staging with CT, MRI and ¹⁸F-FDG-PET/CT combined with surgical white-light visualisation is relatively insensitive for small lesions. Augmenting this staging with intra-operative near-infrared fluorescence imaging for indocyanine green, results in additional metastases being detected in 12-17% of patients undergoing colorectal liver metastasectomy, and can potentially prevent intrahepatic recurrence (Handgraaf et al., 2017).

In Chapter 4, a cohort of resected liver metastases was analysed for clinically relevant morphological tumour features and IHC expression which might be assessed using surrogate biomarkers derived from either routine or experimental

imaging techniques. An important advantage promoted in the attempt to characterise tumour phenotypes by morphological, functional and molecular imaging has been that it is minimally invasive, well tolerated and repeatable (O'Connor et al., 2017). In the future, insights into tumour biology will be acquired non-invasively from analysis of tumour cells, DNA and RNA in the peripheral circulation (Diaz and Bardelli, 2014; Krebs et al., 2014). These techniques are being developed rapidly and are already moving into clinical practice. They too are minimally invasive and repeatable. However, the information that imaging biomarkers can provide, which is likely to remain unique, will be the spatial localisation and phenotypic characterisation of metastatic tumours *in situ*. Phenotypic variation, therefore, will be a key marker for how much additional information tumour characterisation by imaging could provide.

This analysis has highlighted the substantial variation for several important phenotypes. The pattern of tumour-liver interface is heterogeneous in just under half of patients using the Vermeulen classification and this was supported by the results reported by Eefsen et al. (2012). As might be expected, the proportion of patients with within-patient heterogeneity is even higher when patients with a larger number of metastases or recurrent liver metastases (Nielsen et al., 2014), which might make this assessment even more relevant to the polymetastatic/palliative setting. In our cohort, there was a relative high co-incidence of pushing and replacement patterns within the same patients, meaning that there was less heterogeneity for encapsulated (pushing metastases with a fibrous pseudocapsule

and/or metastases with a desmoplastic growth pattern) compared with non-encapsulated metastases (pushing metastases without a fibrous capsule and/or metastases with a replacement growth pattern) (Siriwardana et al., 2016). The concordance of non-fibrotic pushing and replacement pattern metastases between metastases would lend strength to the argument that, while the pattern of angiogenesis, which is through to divide pushing and replacement metastases, is a feature of tumour biology, the fibrotic capsule is due to a protective immune inflammatory response initiated by the liver (Okano et al., 2000), and therefore tends to be consistent within patients. In future work it would be helpful to attempt to understand the mechanisms behind encapsulation, in particular by studying the immune cells at the tumour-liver interface. One of the molecular subtypes of primary colorectal tumours, CSM1, is characterised by microsatellite instability and hypermethylation, with low numbers of somatic copy number alterations (SCNAs) and high expression of genes associated with immune infiltration and immune evasion (Guinney et al., 2015). It would be informative to perform molecular sub-typing of the primary tumours to see whether CSM1 tumours are over or under-presented in the encapsulated group. Towards the end of this project, in collaboration with the Wellcome Trust Centre for Human Genetics in Oxford, I had begun to use FFPE-derived DNA to analyse somatic mutations and SCNA in a subgroup of the metastases, with representation across the subtypes of tumour-liver interface (see Chapter 4).

Chapter 4 also investigated whether patterns of hypoxia and angiogenesis differ between metastases, and a higher expression of GLUT1 in encapsulated metastases in this study corroborated previous findings. It would have been helpful to analyse the MVD in this cohort, rather than only in the smaller prospective subgroup of the FILM study who underwent perfusion imaging, to confirm the finding of Siriwardana et al. (2016), that encapsulated metastases have reduced neovascularisation.

Finally, in Chapter 5, morphological, functional and molecular imaging was correlated with clinically relevant tumour features. This was the first study to provide preliminary data on the potential for morphological MRI to determine encapsulation, but warrants investigation in a larger prospective cohort with optimised imaging. This could be a useful companion biomarker. Based on observations while performing this study, it is also possible that MRI can detect peritumoral vascular invasion, although this will need to be tested in a formal study of diagnostic performance. From the pathological literature, the relative risk of recurrence in patients with venous invasion is approximately 1.77, and this would be a potential contributor to prognostic models based on pre-treatment biomarkers.

It will be challenging to use visual inspection to determine tumour viability based on MRI characteristics, and no evidence was found to support the use of ^{18}F -FDG-PET to determine the proportion of viable cells, although other studies have

shown modest associations in liver metastases treated with chemotherapy (Burger et al., 2013; Dunet et al., 2017). This could have been possibly due to the uncontrolled timing between PET and surgery and/or variations in tumour glucose utilisation per cell. Also, post-treatment ^{18}F -FDG-PET studies might overestimate this correlation relative to treatment naive patients, because of the large alterations in cellular viability in responsive vs non-responsive tumours. However, the subregion methodology based on DCE-MRI developed in this thesis yielded a higher correlation with viability. A limitation of this technique is that it relied on the contrast-time curve developed from more than 20 acquisitions per patient, across at least 7 minutes. Lesion contrast-enhancement pharmacokinetics are routinely used in clinical breast imaging to distinguish benign and malignant tumours based on only five post-contrast acquisitions (albeit the total scanning time is still approximately 6 minutes). It would be useful to analyse whether the contrast-enhancement curves could be accurately described with fewer time points, and still allow acceptable subregion derivation. This would have several advantages. Firstly, clinical liver MRI routinely incorporates whole-liver post-contrast acquisitions at three time-points (designed to optimise lesion identification and characterisation) of tumours and it would be straightforward to implement additional two additional acquisitions to 6-7 minutes to allow the contrast-uptake curve to be drawn. The DCE-MRI sequence used in this study is not compatible with routine clinical scanning, which might limit applicability. Secondly, there would be fewer breath holds for the patient, reducing the burden on the patient and radiographers. Finally, there would be fewer imaging volumes

to motion-correct in post-processing, which could allow more manual supervision of this step, and improve workflow.

There are two major avenues I did not explore in this thesis. Firstly, I did not examine tumour response to chemotherapy. Response to chemotherapy, both on ceCT and ^{18}F -FDG-PET is well-documented to be a good prognostic biomarker in resected oligometastatic CRC (Rubbia-Brandt et al., 2006, Sebagh et al., 2014) as well as polymetastatic CRC (Suzuki et al., 2012). Furthermore the histopathological response within patients is heterogeneous in approximately one fifth of patients within patients (Sebagh et al., 2016), which might be a further role for imaging evaluation of CRLM. Finally, there have been some efforts to use imaging to predict response of CRM to chemotherapy, although the evidence for this is limited (Beckers et al., 2017). However, the larger retrospective cohort I studied was not part of a prospective study which meant that the timing of imaging relative to neoadjuvant treatment was not controlled, nor was the type, timing and duration of chemotherapy consistent. I felt this may have potentially given rise to significant additional heterogeneity in the imaging variables and clinical outcomes, for which would compromise interpretation.

This thesis did not investigate data-driven, as opposed to hypothesized, approaches to analysing the available imaging data. There is currently a significant focus on adopting radiomic approaches to imaging data in the search for predictive or prognostic biomarkers (Aerts et al., 2014; Gillies, Kinahan, Hricak,

2016). This involves extracting numerous quantitative outputs, typically from CT, although it can be applied to any imaging data. This was for two reasons: a) I did not have access to the engineering expertise to extract and analyse high dimensional imaging data and b) I was concerned that in the relatively small cohort I studied, there would be statistical challenges with analysing hundreds of imaging variables.

In the future, growing clinical and scientific interest in this area of practice will drive developments in treatment. Improvements in local treatment techniques and systemic therapies will render more patients with metastatic cancer suitable for treatment with radical intent. This is likely to translate into better outcomes. A major challenge for this field is grounding clinical practice in controlled clinical trials. This will allow emerging treatments and treatment strategies to be trialled against current best practice, but also will provide a backdrop in which predictive and prognostic biomarkers can be prospectively tested. As discussed throughout this thesis, imaging biomarkers currently contribute to the clinical selection of patients for radical therapy and, due to the potential to repeatedly assess and localise multisite disease, are likely to remain relevant in the metastatic setting. Improvements in the sensitivity of imaging techniques are likely to continue to evolve alongside technological advances, and therefore further refine the imaging assessments of disease burden, which are central to their current use in prognostic models. In comparison, imaging biomarkers based on disease characterisation have been relatively under-investigated in CRLM. It has always

been challenging to conduct studies using novel or non-standard imaging techniques in sufficient numbers to become robust biomarkers alongside routinely assessed clinical biomarkers, or histopathological biomarkers which can be revisited from banked tissue. Blood-based biomarkers are likely to further impact on the potential for imaging to remain competitive in a crowded and poorly defined space. I believe the strategy adopted in this thesis, linking imaging to tumour biology, is likely to remain the most important step. Leveraging modern computing power to provide reliable and repeatable (radiomic) imaging variables from conventional, routinely acquired imaging is likely to be an important component of the effort to link imaging to tumour biology and outcome. It is important that the required imaging substrate (conventional or novel) from which biomarkers can be prospectively and retrospectively derived, is routinely and consistently acquired alongside prospective interventional trials, which has become routine for tissue banking in oncology trials.

Future directions for research

i. Chapter 3

1. The analysis in this study had a number of limitations, which were described above, in particular the suboptimal characterisation of cohort for non-imaging parameters and the small sample size, which limited the meta-analysis. The next step in testing these putative biomarkers and their added prognostic value would be to test and validate them in large, well-described cohorts. A group in Liverpool, UK have studied a cohort of 564 patients and examined the impact of RAS status on traditional clinical scoring systems (Brudvik et al., 2016). I have previously collaborated with this group (Jones et al., 2017), and have been invited to develop the ^{18}F -FDG-PET biomarkers described in this study in this larger cohort of patients.
2. The finding that background liver SUV may have prognostic implications is interesting. Further investigation to confirm if this is a significant finding in a larger cohort is warranted, alongside an investigation of the biological process underlying this imaging feature. It would also be interesting to establish if elevated liver FDG-uptake is related to liver-specific changes, or to a systemic inflammatory response. To investigate this, I would want to correlate the background liver SUV to changes in serum inflammatory markers, such as serum CRP, WCC, PLR, NLR and albumin, in a retrospective cohort. I would also derive the bone marrow FDG-uptake from the existing dataset and correlate with the liver uptake to assess if these are related phenomena, which

might imply a systemic process. To investigate if this is a liver-specific phenomena, I would correlate the background liver SUV with serum transaminases and would derive heterogeneity statistics for the background liver parenchyma on the attenuation correction CT performed at the time of the PET (e.g. Ganeshan et al., 2011).

3. It may be that changes in background liver SUV are prognostically relevant in primary, non-metastatic CRC. In our local practice, there are subgroups of patients with CRC who receive ^{18}F -FDG-PET as part of their staging investigations, for example due to the presence of poor prognostic features or involvement in clinical trials. I would derive the background liver ^{18}F -FDG-PET data for these patients which could then be analysed and compare with patient outcomes.

ii. Chapter 4

1. The presence of a fibrous capsule seems to be prognostically and therapeutically important, and an improved understanding of this phenomenon would be valuable. I would extend the analysis to assess MVD in the entire cohort, to confirm the previous finding that there is relatively more neoangiogenesis in non-encapsulated metastases. This might help support the use of an imaging biomarkers for encapsulation as a companion biomarker in studies of antiangiogenic agents. Secondly, I would like to look at strategies to analyse the inflammatory profile at the liver-tumour interface, and compare this with the pattern of encapsulation.

2. Although this was beyond the scope of this thesis, I had begun analysing SCNA in subsets of the analysed metastases with representatives from all three of the Vermeulen (and both Siriwardana) patterns of liver-tumour interface (see Chapter 4). It would be interesting to test whether there is an association between the pattern or burden of SCNA and the presence of encapsulation.

iii. Chapter 5

1. As discussed above, segmentation of metastases might be feasible on the basis of an extended conventional liver MRI protocol, which significantly extend its clinical applicability. I would perform an analysis to establish whether the segmentation derived from the contrast-uptake curves was robust for a more limited dataset of 5-7 post-contrast acquisitions which could be incorporated into routine liver MRI protocols.
2. A major limitation of MRI to detect histological features is the spatial resolution, in combination with motion artefact which will tend to obscure smaller structures. Limited attempts with existing clinical sequences did not improve spatial resolution. I did not have access to MRI physics expertise to develop high-resolution, smaller field of view imaging for lesional, rather than whole liver, assessment during this project, but that would be a goal of future research.

Appendix 1: Imaging and histology

Table A1a: Imaging and histology available in the retrospective cohort

	NAC	MRI		CT		PET		Histology	
Patient		Pre	Post	Pre	Post	Pre	Post	Metastasis	Primary
CRLM001	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM002	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
CRLM003	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM004	No	Yes	NA	Yes	NA	Yes	NA	Yes	No
CRLM005	No	Yes	No	Yes	No	Yes	No	Yes	No
CRLM006	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM007	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM008	No	Yes	NA	Yes	NA	No	NA	No	No
CRLM009	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM010	Yes	No	No	Yes	Yes	Yes	No	No	No
CRLM011	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM012	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM013	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM014	No	No	NA	Yes	NA	No	NA	No	No
CRLM015	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM016	Yes	Yes	No	Yes	Yes	Yes	No	Yes	No
CRLM017	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
CRLM018	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM019	Yes	No	No	Yes	Yes	No	No	Yes	Yes
CRLM020	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM021	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CRLM022	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM023	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No
CRLM024	No	Yes	NA	Yes	NA	Yes	NA	Yes	No
CRLM025	Yes	Yes	No	Yes	Yes	Yes	No	Yes	No
CRLM026	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM027	No	No	NA	Yes	NA	Yes	NA	No	No
CRLM028	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
CRLM029	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM030	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CRLM031	No	Yes	NA	Yes	NA	Yes	NA	Yes	No
CRLM032	Yes	No	No	Yes	Yes	Yes	Yes	No	No
CRLM033	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM034	Yes	No	Yes	Yes	Yes	Yes	No	Yes	No
CRLM035	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM036	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM037	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM038	No	Yes	NA	Yes	NA	Yes	NA	Yes	Yes
CRLM039	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM040	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
CRLM041	No	Yes	NA	Yes	NA	Yes	NA	Yes	Yes
CRLM042	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM043	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No

Patient	NAC	MRI		CT		PET		Histology	
		Pre	Post	Pre	Post	Pre	Post	Metastasis	Primary
CRLM044	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM045	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM046	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No
CRLM047	No	Yes	NA	Yes	NA	Yes	NA	Yes	No
CRLM048	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CRLM049	Yes	Yes	No	Yes	Yes	Yes	No	No	No
CRLM050	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM051	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM052	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM053	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM054	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM055	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CRLM056	Yes	No	Yes	Yes	Yes	No	Yes	No	No
CRLM057	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM058	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM059	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM060	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM061	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM062	No	Yes	No	Yes	No	Yes	No	Yes	No
CRLM063	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM064	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
CRLM065	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No
CRLM066	Yes	Yes	Yes	Yes	Yes	No	No	No	No
CRLM067	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No
CRLM068	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM069	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
CRLM070	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM071	No	Yes	NA	Yes	NA	Yes	NA	Yes	Yes
CRLM072	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM073	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
CRLM074	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM075	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM076	No	Yes	NA	Yes	NA	Yes	NA	Yes	No
CRLM077	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CRLM078	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM079	No	Yes	NA	No	NA	Yes	NA	No	No
CRLM080	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM081	Yes	No	Yes	Yes	Yes	Yes	No	No	No
CRLM082	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM083	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM084	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
CRLM085	No	Yes	NA	Yes	NA	Yes	NA	No	No
CRLM086	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
CRLM087	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Notes

NAC = neoadjuvant chemotherapy. Pre and post refer to pre- and post-NAC. NA = not applicable.

Appendix 2: Individual patient variables

Table A2a: Selected patient variables used in the retrospective cohort study

	Primary*			DFI®	#	Bilobar	Size	EHD	SUV	SUV	Background	hPFS	DFS
				(days)	mets		(mm)		max	mean		(days)	(days)
Patient	C/R	T	N										
CRLM001	C			541	1	0	15	0	11.10	5.75	3.36	1155	1155
CRLM002	C	3	0	12	3	0	33	0				95	95
CRLM003	C	3	0	0	1	0	11	0	4.98	3.42	2.72	727	727
CRLM004	R	2	2	13	5	0	23	0	18.20			543	543
CRLM005	R	3	2	27	1	0	33	0	4.60	4.66	1.85	433	433
CRLM006	C	4	2	407	4	1	7	0	7.64	5.21	3.77	99	99
CRLM007	C			0	1	0	35	0	5.94	3.75	2.74	853	853
CRLM008	C			8	1	0	20	0				1817	1817
CRLM009	C	4	2	0	1	0	50	0	8.75	5.25	2.81	792	792
CRLM010	C			0	2	0	40	0	16.33	8.71	4.05	36	36
CRLM011	R	3	0	440	9	1	17	0	16.33	8.75	4.11	110	110
CRLM012	C		0	656	2	0	30	0	11.36	5.5	3.73	763	763
CRLM013	R	3	1	115	4	1	13	1	8.30	3.9	2.4	307	0
CRLM014	R	3	2	8	3	0	88	0				1640	1640
CRLM015	C	3	0	7	2	0	68	0	10.49	5.68	2.95	1694	1694
CRLM016	C			1341	1	0	99	0	23.50	7.58	3.62	1636	1636
CRLM017	C	4	2	0	4	1	25	1	14.20	6.96	3.32	168	168
CRLM018	C			735	1	0	38	1	10.80	5.6	3.38	456	0
CRLM019	C	3	1	0	10	1	100	0				167	167
CRLM020	R	3	1	817	1	0	27	0	9.60	5.21	2.96	638	422
CRLM021	R	3	2	194	5	1	15	0	12.40			401	401
CRLM022	C	3	1	0	7	1	30	1				104	0
CRLM023	R	4	2	15	1	0	42	0	12.51	5.37	2.74	140	140
CRLM024	C	3	2	260	1	0	25	0	9.91	5.71	3.45	271	271
CRLM025	C	3	2	12	5	1	42	0	17.47	7.38	3.33	243	243
CRLM026	C	4	1	0	1	0	15	0	10.90	5.49	3.41	1707	1707
CRLM027	R	3	2	380	1	0	17	0	9.21	5.24	3.04	1091	1091
CRLM028	R	3	1	7	2	0	67	0	9.95	5.75	2.46	117	117
CRLM029	C	3	2	223	2	0	10	0	7.07	4.32	2.89	236	236
CRLM030	C	4	1	10	1	0	50	0	7.67	4.6	2.92	430	115
CRLM031	C			0	2	0	45	0	10.15	4.15	2.46	308	308
CRLM032	C	4		4	1	0	10	0	5.43	4.6	3.65	404	404
CRLM033	R	3	0	392	2	0	16	0	12.45	5.67	2.72	1009	1009
CRLM034	R	3	0	817	1	0	30	1	5.34	3.39	2.31	1560	1560
CRLM035	C	3	0	0	1	0	27	0	10.54	4.58	2.38	474	474
CRLM036	C			88	1	0	10	0	6.69	4.08	2.75	413	413
CRLM037	C			65	7	0	10	0	5.53	4.96	4.16	421	421
CRLM038	R	2	0	721	2	0	56	0	8.12	4.58	2.8	1440	1440
CRLM039	R	2	0	757	2	0	23	0	5.40			424	424
CRLM040	R	3	2	5	2	0	45	0	15.07	5.92	2.8	1171	660
CRLM041	C	3	1	759	2	0	30	0	14.68		3.63	691	691
CRLM042	R	2	0	421	2	0	10	0	6.06	3.65	2.47	1142	1142
CRLM043	R	3	1	1	1	0	25	0	3.71	3.00	2.46	817	817
CRLM044	C	3	0	448	1	0	35	0	16.46	7.38	2.75	1475	419
CRLM045	C	4	1	0	2	0	24	0				428	428
CRLM046	R	4	2	6	5	0	12	0	5.72	3.91	2.8	104	104
CRLM047	C	3	1	1266	2	1	70	0	9.66	5.45	2.45	396	396
CRLM048	R	3	1	502	2	0	35	1	8.58	4.45	2.06	56	56

Primary*				DFI®	#	Bilobar	Size	EHD	SUV	SUV	Background	hPFS	DFS
					mets				max	mean			
Patient	C/R	T	N	(days)			(mm)					(days)	(days)
CRLM049	C	3	0	398	4	0	35	0	20.08	6.1	1.98	798	798
CRLM050	R			2385	1	0	41	0	10.60	5.42	2.84	1265	1265
CRLM051	C			0	1	0	13	0	3.97	3.15	2.43	176	176
CRLM052	C			136	2	0	25	0	6.09	4.09	3.05	164	164
CRLM053	C	4	2	0	4	1	55	0	11.57	6.03	2.85	82	82
CRLM054	R	3	1	13	4	1	20	0	9.78	5.58	3.47	493	493
CRLM055	C	3	2	0	4	1	40	0	12.39	5.48	2.57	1233	1233
CRLM056	C	4	1	0	1	0	30	0				206	206
CRLM057	C	3	2	3	4	1	29	0	8.62	2.86	2.17	1234	1234
CRLM058	R	3	0	1	2	1	26	0	9.88	5.26	2.83	191	191
CRLM059	R	4	2	0	2	0	20	0				1098	1098
CRLM060	R	3	1	778	1	0	25	0	10.69	5.2	2.69	1027	1027
CRLM061	C			1113	1	0	35	0	4.01	.	3.6	1254	1254
CRLM062	C	4	2	343	1	0	55	0	8.83	4.53	3.19	47	47
CRLM063	R	4	2	8	9	1	17	0	19.11	6.94	3.4	34	34
CRLM064	R	3	1	13	1	0	20	0	14.82	6.64	3.2	357	357
CRLM065	C			133	3	0	10	0	15.67	4.82	2.48	201	201
CRLM066	R	3	2	5	10	1	20	0				1129	1129
CRLM067	R	3	1	12	1	0	39	0	7.77	4.81	2.9	1139	1139
CRLM068	C			14	3	1	45	0	15.70	7.06	2.65	77	77
CRLM069	R	4	2	2	1	0	77	0	21.28	5.12	2.23	1007	1007
CRLM070	R	3	1	0	1	0	14	0	6.24	4.77	3.61	177	177
CRLM071	R	3	1	1196	1	0	21	0	8.58	5.37	3.53	1038	1038
CRLM072	C	3	2	85	4	0	10	0	7.78	3.83	2.62	536	536
CRLM073	R	4	1	0	2	0	5	0	9.72	4.94	3.14	102	102
CRLM074	C	4	1	142	3	0	14	0	4.95	3.94	3.24	253	253
CRLM075	C	3	1	254	2	0	17	0	15.55	5.6	2.66	636	636
CRLM076	R			450	1	0	14	0	3.40	3.24	2.82	223	223
CRLM077	C	4	0	0	3	0	22	0	9.20	4.59	2.67	819	819
CRLM078	C			572	1	0	17	0	4.70	3.91	3.13	260	260
CRLM079	C	4	2	0	1	0	30	0	5.40	4.3	3.6	730	730
CRLM080	R	3	2	32	3	0	15	0	6.07	4.39	3.28	359	359
CRLM081	C	3	2	0	2	0	17	0	3.50			811	811
CRLM082	C			0	5	1	58	0	11.09	5.97	2.58	152	152
CRLM083	C	4	2	0	1	0	78	1	16.70	7.52	3.46	622	0
CRLM084	C	3	1	37	5	0	22	0	11.50	4.92	3.15	698	130
CRLM085	R	1	0	445	1	0	24	0	13.70	7.46	3.96	722	722
CRLM086	R	4	2	138	1	0	26	1	11.11	5	2.2	308	0
CRLM087	R	3	2	5	1	0	38	0	17.30	6.03	2.29	47	47
FILM001	C	3	0	1	4	0	18	0	16.25	5.05	2.49	574	574
FILM002	C	3	2	774	1	0	16	0	9.12	5.05	2.77	321	191
FILM003	R	1	0	630	2	0	13	0	6.06	4.18	2.85	0	0
FILM004	R	2	0	511	1	0	16	0	6.71	4.23	2.91	0	0
FILM005	R	3	1	848	1	0	32	0			3.08	497	497
FILM006	R	3	1	719	1	0	30	0	11.79	6.24	3.67	196	196
FILM007	R	3	0	402	1	0	35	0	10.87	5.5	2.59	196	196
FILM008	R	4	0	236	1	0	40	0	4.58	3.13	2.54	173	173
FILM010	C	3	1	0	2	1	16	0	14.7	7.72	3.67	0	0

Notes

*Features of primary tumour: (C)olonic vs (R)ectal, T-stage and N-positive. @DFI = disease-free interval. EHD = extra-hepatic disease.

Appendix 3: pCT protocol

Patient preparation:

- This scan requires administration of IV contrast via at least a 20G cannula sited in the antecubital fossa and flushed prior to use.

Hardware:

- 64-slice GE CT (LightSpeed VCT, GE Healthcare, Amersham, UK)

Oral and IV contrast:

- No oral contrast.
- 70mL of IV iohexol (Omnipaque 300; GE Healthcare, Milwaukee, WI, USA) delivered at 5mL/s using a pump injector followed by a 20mL saline flush.

Imaging acquisition:

- Images acquired in 3 phases:
 - Pre-contrast to cover entire liver
 - 2.5mm contiguous helical block in end inspiration
 - Pitch 0.984:1
 - Rotation time = 0.5s
 - 120kV
 - Automated + smart mA (100-700)
 - Noise index = 32

- Perfusion imaging
 - Plan 4cm craniocaudal coverage based on pre-contrast acquisition to cover lesion of interest.
 - Start IV pump injection
 - 10s delay. 45s 5mm cine acquisition in end inspiration
 - Rotation time = 1s
 - 8i per rotation
 - 120kV
 - 60mA
 - Noise index N/A
- PV phase
 - 0.625mm contiguous helical block in inspiratory phase
 - Pitch 0.969:1
 - Rotation time 0.5s
 - 120kV
 - Automated + smart mA (100-700)
 - Noise index = 32

Appendix 4: DCE-MRI protocol

Patient preparation:

- This scan requires administration of IV contrast via at least a 20G cannula sited in the antecubital fossa and flushed prior to use.
- Supine. Feet first.

Hardware:

- GE Healthcare Signa HDx Twinspeed 1.5T
- 8 channel body coil

Oral and IV contrast:

- No oral contrast.
- 15mL 0.5mmol/mL IV gadoteridol (ProHance; Bracco Diagnostics, Princeton, NJ, USA) delivered at 3mL/s using a pump injector followed by a 15mL saline flush.
Second bolus delivered for AIF.

Acquisitions:

- 3-plane planning acquisition (2D gradient echo)
- Coronal SSFSE (coronal T2W sequence)
- Axial fsFRFSE (axial T2W sequence with fat saturation)
- Axial DWI (with multiple B values (B500/250/100/50/0))
- T2* practice (sagittal or coronal spoiled gradient echo)

Use of applicant and project information

As a consequence of your application, you will now be registered as a member of the CRUK Oxford Centre and will have your project title added to the CRUK Oxford Centre website (www.cancercentre.ox.ac.uk). If, in your application, you requested discussion before the publishing of this information the Centre Manager will be in touch. The CRUK Oxford Centre will require a report once a project is completed which details the outputs and achievements of the work. Information regarding successful awards may also be shared with Cancer Research UK as the provider of the funding of the CRUK Oxford Centre.

Further information

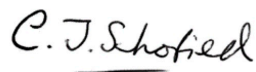
As the award is derived from CRUK funding, these funds must be in line with CRUK Terms and Conditions of grant awards, available at: <http://science.cancerresearchuk.org/funding/terms-conditions-and-policies/index.htm>

Any queries on the terms and conditions of the award may be addressed to CRUK Oxford Centre Manager who may be contacted by email at cancercentre@oncology.ox.ac.uk.

Accounting arrangements

The award will be transferred via RPF3 to your Department. Please contact Jackie Fellows (jackie.fellows@oncology.ox.ac.uk) for more information regarding financial information.

Yours faithfully



Professor Chris Schofield

Deputy Chair of the CRUK Oxford Centre Research Committee

Acceptance of Terms and Conditions of Award

Please sign and date below to confirm acceptance of the terms and conditions of the Development Fund Award as detailed above. When signed please send to cancercentre@oncology.ox.ac.uk alongside your Award Reference shown on page 1. An RPF3 of funds will be initiated upon receipt of the signed acknowledgement.

Signature of grant awardee: _____ Title: _____

Signature of Departmental Administrator: _____ Title: _____

Date: _____

Financial 2/3 code for grant transfer: _____

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- Axial multiple slip angles (15/9/3) for T1₀
- Axial LAVA (T1W sequence with fat saturation)
- Axial Multiphase LAVA (DCE-MRI)

- 3D
- FOV = 42 (same as planning and T1₀ sequences)
- Phase FOV = 0.8
- Matrix = 320 x 192
- Slice Thickness = 5.0
- Location per slab = 22
- Overlap = 0
- TR = 4.46s
- TE = 2.128s
- Acquire images in end expiration.
 - 0-2 minutes – single breath between acquisitions
 - 204 minutes – two breaths between acquisitions
 - 4-6 minutes – three breaths between acquisitions
 - 6-7 minutes – four breaths between acquisitions
- Start contrast injection midway through third acquisition
- Manually record timepoints
- Axial LAVA (whole liver)
- Coronal T2* of aorta (100s acquisition. Start second bolus injection at the start of imaging)

Appendix 5: DCE-MRI timings

Table A5a: dceMRI timings for the FILM study

Patient	Acquisition number																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
FILM001*	0	13	25	41	55	67	79	93	108	129	147	168	189	213	238	256	282	300	321	346	371	397	425	450	473					
FILM001%	0	15	32	50	65	84	100	116	137	160	178	200	224	246	276	306	336	364	400	436										
FILM002	0	14	28	43	57	71	85	100	114	128	142	157	175	194	210	228	250	271	290	312	334	358	384	407	435					
FILM003	0	13	27	42	56	74	89	104	121	142	158	176	197	216	236	261	289	318	349	384	415									
FILM004	0	14	27	41	54	69	82	96	110	124	142	162	181	201	219	236	258	279	301	320	340	363	381	402	424					
FILM005	0	11	25	37	50	62	74	85	98	110	127	144	160	178	195	210	226	244	265	288	308	328	350	370	392	414				
FILM006	0	11	22	35	46	57	67	75	87	99	109	120	133	145	158	170	184	197	209	222	237	254	271	288	304	322	339	357	378	398
FILM007	0	11	25	38	51	63	76	90	104	117	135	153	170	187	204	221	238	256	278	300	321	342	362	387	412	438				
FILM008	0	10	22	35	49	64	77	91	103	116	135	154	173	192	211	229	248	272	295	316	338	360	388	415	441					
FILM010*	0	15	30	45	59	75	92	109	127	145	169	191	211	234	257	284	309	334	361	389										
FILM010%	0	15	28	43	57	72	85	100	114	134	155	175	196	217	239	267	294	320	346	371	395	421								
FILM011	0	15	29	45	61	77	93	109	125	141	164	185	208	231	259	287	316	342	369	401	427									
FILM012	0	14	28	40	53	66	78	91	104	118	136	152	169	188	205	223	240	258	279	300	322	344	365	389	413	422				
FILM013	0	15	29	44	58	72	90	102	119	135	149	165	183	197	212	232	253	271	298	321	342	367	392	420						
FILM014	0	14	28	42	56	69	85	104	116	132	153	176	195	217	241	261	278	304	329	349	372	399	421							
FILM015	0	13	27	41	54	67	80	93	107	121	139	159	178	198	218	263	288	313	337	360	387	416	446							

Notes

Numbers are seconds after the start of scanning.

Contrast injection started halfway through the **third** acquisition.

Italics = breathed during acquisition

Contrast injected halfway through third scan

* = pre neoadjuvant chemotherapy

% = post neoadjuvant chemotherapy

Appendix 6: Pathological assessment

Metastases Assessment			Study ID	FILM	
Assessor Initials		Date			20
		LESION 1	LESION 2	LESION 3	
Resection margin (if clear – distance in mm)	Involved				
	Clear (mm)				
Size (mm)	X				
	*	*	*	*	
	Y				
	*	*	*	*	
Margin definition	Z				
	Well				
	Intermediate				
	Poorly				
Shape: lobulated (L) vs spherical (S) vs irregular (Ir)					
Differentiation	Well/Moderate				
	Poorly				
Mucinous (if yes - %)	No				
	Yes - %				
Calcification (✓/X)					
Fibrous capsule ⁱⁱⁱ	No				
	Thin				
	Thick				
Compressed peripheral hepatocytes	No				
	Yes – thickness (mm)				
Tumour necrosis (if yes - % necrosis)	No				
	Yes - %				
Tumour fibrosis (If yes - % fibrosis)	No				
	Yes - %				
Percentage of Viable Cells (Blazer ^{iv}) 1 block per centimeter of nodule ^v	0%				
	1-49%				
	50%+				
Perineural invasion (✓/X)					
Vascular invasion (✓/X) CD34 stain	No				
	Arterial				
	Portal				
Biliary invasion (✓/X)					
Micrometastases (✓/X)	No				
	Parenchymal				
	Portal				
Tumour thickness at tumour-normal interface ^{vi} (mm)					
Tumour Regression Grading (Rubbia-Brandt ^{vii})	1				
	2				
	3				
	4				
	5				

Appendix 7: Funding Letters

OHSRC

Oxfordshire Health Services Research Committee

Our ref: EC/955

Dr James M Franklin
Department of Radiology
Churchill Hospital
Headington
Oxford
OX3 7LJ

Elaine Cherry
R&D Office
Manor House
John Radcliffe Hospital
Headley Way, Headington
Oxford OX3 9DZ
Tele: (01865) 222 147
Fax: (01865) 222 648
Elaine.cherry@orh.nhs.uk

5 November 2009

Dear Dr Franklin

**OXFORDSHIRE HEALTH SERVICES RESEARCH COMMITTEE (OHSRC)
RESEARCH PROJECT: 955**

The assessment of colorectal liver metastases by perfusion CT, dynamic contrast enhanced MRI and FDG-PET: their relationships, prognostic value and correlation with tumour biology.

Your application for funding for the above project has been considered by members of the Oxfordshire Health Services Research Committee, and a grant of £6,575.40 has been agreed.

Please contact Mrs Sue Derham, Charitable Funds, Manor House, John Radcliffe Hospital, Headington, Oxford, OX3 9DZ, Tel: (01865) 222719, to obtain the cost centre to be quoted on all requests for payment and to discuss the nature and timing of the expenses you outline, particularly payment of any staff. Please have your Ethics reference number and approval date ready, which will be required before any funds are released. The award is subject to ethical approval, if appropriate, being in place.

If your grant covers equipment, any medical and scientific equipment (and some consumables) purchased from the fund may be exempt from VAT, as the OHSRC funds come from the Oxford Radcliffe Hospitals Charitable Fund, which is a Registered Charity. When ordering such equipment, you should speak directly to Mrs Sue Derham, Charitable Funds (contact details above) to establish whether it is exempt from VAT. When the equipment has been received, the certified and coded invoices should be sent to: Mrs Sue Derham, for payment. The equipment becomes the property of the host Trust and will remain thus at the end of the research.

The project must be started within 12 months of the grant being awarded. Any delays must be reported immediately. In addition, if the grant is not used within two years, then the funding will lapse unless an adequate explanation can be provided and agreed.

Annual updates of the progress of the research and a report of the outcome are required. (A reminder letter will be sent when these reports are due). I wish you every success with this study.

Yours sincerely

Professor John Stradling
Chairman, Oxfordshire Health Services Research Committee

cc: Mrs Sue Derham, Charitable Funds, John Radcliffe Hospital



CANCER
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OXFORD
CENTRE



CRUK Oxford Centre
University of Oxford, Department of Oncology
Old Road Campus Research Building
Roosevelt Drive
Oxford
OX3 7DQ

Monday 20th July 2015

Dear Dr Franklin,

Award from the CRUK Oxford Centre Development Fund

Thank you for your application to the CRUK Oxford Centre Development Fund in May 2015. I am pleased to inform you that the CRUK Oxford Centre Research Committee has agreed at their meeting on 10 July 2015 to allocate funds to your project.

Project Title: Genomic characterisation of imaging phenotypes in colorectal liver metastases

Amount awarded: £14,972.15

Project dates: Start- 01/09/2015 End- 31/08/2016

Award Ref: CRUKDF 0715 - JF

Reporting Requirements

The CRUK Oxford Centre has to ensure that the best possible use is made of allocations from the Development Fund. The Research Committee will monitor all allocations made from the fund, and so you will be required to submit a brief report on how the project for which funds have been awarded has met the research objectives and expected outcomes which were set out in your application.

Requirements relating to start of project

It is a condition of this award that expenditure should be incurred up to the end date defined above, unless an extension is granted; applicants should write to the CRUK Oxford Centre Manager within a month of the start date, if they wish to request such an extension. It is also a condition of this award that any unspent funds, once all grant costs have been met, be returned to the CRUK Oxford Centre Development Fund, for the benefit of future applicants. For any future applications to the Development Fund a report and financial reconciliation must be received for the current award.

Acknowledgement of CRUK Oxford Centre Development Fund Support

Where a publication or oral/poster presentation arises from a project supported by the CRUK Oxford Centre, it is a condition of the award that you should acknowledge the support of the Development Fund using the following "This work was supported by Cancer Research UK (CR-UK) grant number C5255/A18085, through the CRUK Oxford Centre". Please note this grant code may change, updated language will be provided when available. The CRUK Oxford Centre and CRUK logos are available through the Centre Operations Team on cancercentre@oncology.ox.ac.uk, for prominent display in all poster and oral presentations.

CRUK Oxford Centre, Department of Oncology | +44 (0) 1865 617043 | www.cancercentre.ox.ac.uk

Use of applicant and project information

As a consequence of your application, you will now be registered as a member of the CRUK Oxford Centre and will have your project title added to the CRUK Oxford Centre website (www.cancercentre.ox.ac.uk). If, in your application, you requested discussion before the publishing of this information the Centre Manager will be in touch. The CRUK Oxford Centre will require a report once a project is completed which details the outputs and achievements of the work. Information regarding successful awards may also be shared with Cancer Research UK as the provider of the funding of the CRUK Oxford Centre.

Further information

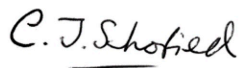
As the award is derived from CRUK funding, these funds must be in line with CRUK Terms and Conditions of grant awards, available at: <http://science.cancerresearchuk.org/funding/terms-conditions-and-policies/index.htm>

Any queries on the terms and conditions of the award may be addressed to CRUK Oxford Centre Manager who may be contacted by email at cancercentre@oncology.ox.ac.uk.

Accounting arrangements

The award will be transferred via RPF3 to your Department. Please contact Jackie Fellows (jackie.fellows@oncology.ox.ac.uk) for more information regarding financial information.

Yours faithfully



Professor Chris Schofield

Deputy Chair of the CRUK Oxford Centre Research Committee

Acceptance of Terms and Conditions of Award

Please sign and date below to confirm acceptance of the terms and conditions of the Development Fund Award as detailed above. When signed please send to cancercentre@oncology.ox.ac.uk alongside your Award Reference shown on page 1. An RPF3 of funds will be initiated upon receipt of the signed acknowledgement.

Signature of grant awardee: _____ Title: _____

Signature of Departmental Administrator: _____ Title: _____

Date: _____

Financial 2/3 code for grant transfer: _____

Having originally developed the protocol for the FILM Study, and gained ethical approval prior to my CRF, I subsequently amended the protocol in 2014/15, preparing all the relevant documents on behalf of the PI, Professor Fergus Gleeson.

Copy to: Heather House, Oxford University Hospitals NHS Trust

Tel: 0117 3421389

A Research Ethics Committee established by the Health Research Authority

NRES Committee South Central - Berkshire

Bristol Research Ethics Committee Centre
Whitefriars
Level 3 Block B
Levens Mead
Bristol
BST 2NT

Telephone: 0117 342 1389

18 February 2015

Professor F V Gleeson
Oxford University Hospitals NHS Trust
The Churchill Hospital, Old Road
Headington
OX3 7LJ

Dear Professor Gleeson

Study title: The assessment of colorectal liver metastases by dynamic imaging: the relationship between imaging modalities and correlation with tumour biology.

REC reference: 11/SC/0250
Protocol number: FILM/2011/001
Amendment number: Modified Substantial amendment 1
Amendment date: 12 October 2014
IRAS project ID: 36592

Thank you for submitting the above amendment, which was received on 10 February 2015. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 12 December 2014 refers).

The modified amendment has been considered on behalf of the Committee by the Chair and Vice-Chair.

Ethical opinion

The Chair and Vice Chair stated that only genetic samples obtained with the appropriate consent (i.e. participants who had re-consented or newly consented participants) could be provided for analysis. Any additional cohorts could be seen as fishing and would warrant a new application.

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Letters of invitation to participant [FILM Study Patient Invitation Letter Lung]	1.0	05 December 2013
Letters of invitation to participant [FILM Study Patient Invitation Letter Liver]	1.1	05 December 2013
Notice of Modified Amendment	Modified Substantial amendment 1	12 October 2014
Other [Summary of Changes FILM]	1.0	12 October 2014
Other [Unfavourable Opinion Letter]		12 December 2014
Other [Substantial Amendment Valid Letter]		19 November 2014
Other [Response Letter]		13 February 2015
Participant consent form [FILM Study Consent Form v2.0 101014]	2.0	10 October 2014
Participant information sheet (PIS) [FILM Study Patient Information Sheet Lung]	1.0	05 December 2013
Participant information sheet (PIS) [FILM Study Patient Information Sheet Liver]	2.0	12 October 2014
Research protocol or project proposal [FILM Study Protocol v2.0]	2.0	12 October 2014

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at <http://www.hra.nhs.uk/hra-training/>

11/SC/0250:

Please quote this number on all correspondence

Yours sincerely

pp
Mr David Carpenter
Chair

E-mail: nrescommittee.southcentral-berkshire@nhs.net

Copy to: Heather House, Oxford University Hospitals NHS Trust

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