

**Using functional imaging to improve chemoradiotherapy outcomes
in pancreatic and rectal cancer: moving towards a hypoxia targeting
approach**

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Abstract

Biologically individualised, adaptive radiotherapy requires the integration of information obtained from molecular biomarkers with functional imaging to inform high-precision radiotherapy planning. Functional imaging allows the non-invasive assessment of heterogeneity in a number of tumour physiological characteristics, including the well-documented cause of chemoradioresistance, hypoxia. Functional imaging may be useful in treatment strategy selection and biological target volume (BTV) definition for selective radiotherapy dose escalation. Studies in a number of tumour sites suggest that the response in functional imaging parameters offers more predictive and prognostic information than single time-point assessments, including heterogeneity in tumour reoxygenation in head and neck cancer. In this thesis, the role of functional imaging was investigated in two tumour types. Data from the ARC-II clinical trial in locally advanced pancreatic cancer (LAPC) was used and a new trial in rectal cancer (RHYTHM) was developed and opened.

Chemoradiotherapy (CRT) outcomes in LAPC are poor, with a median overall survival of around 15 months. The benefit of definitive CRT over chemotherapy alone in LAPC is controversial despite 30% of patients with pancreatic cancer only ever having locally progressive disease. The utility of pre- and post-chemoradiotherapy ^{18}F -fluorodeoxyglucose (FDG) positron emission tomography (PET)-derived parameters in treatment strategy selection and patient prognosis was assessed. The limitations of using FDG-PET for BTV definition were explored. A method of characterising hypoxia using ^{18}F -fluoromisonidazole (FMISO) PET was developed that demonstrated the hypoxic BTV to remain spatially consistent with time. The ability of nelfinavir to modulate hypoxia and perfusion was then assessed using dynamic FMISO-PET and perfusion computed tomography (pCT).

While approximately 75% of patients with locally advanced rectal cancer can be successfully cured, the current treatment paradigm includes CRT followed by total mesorectal excision (TME) with its associated long-term morbidity. If pathological complete response (pCR) could be accurately identified on post-CRT imaging and if rates of pCR could be improved upon, organ-preserving strategies may be adopted. Current anatomical imaging techniques recognise poor treatment responses well, but under-report pCR. The ability of a CT texture feature approach to pCR prediction was investigated. Imaging, tissue and circulating biomarkers of hypoxia (FMISO-PET, pimonidazole and carbonic anhydrase IX [CAIX] immunohistochemistry of rectal cancer tissue and osteopontin) and perfusion (pCT, dynamic contrast-enhanced magnetic resonance imaging [dce-MRI], CD31 immunohistochemistry) as well as RNA sequencing of rectal cancer tissue were assessed in a clinical study to identify the best method of characterising the modulation of hypoxia and perfusion in rectal cancer during fractionated CRT. Early analyses of trial data will be discussed. Trial recruitment is ongoing.

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List of abbreviations

µm	micrometre
µmol	micromole
2D	two-dimensional
3D	three-dimensional
4D	four-dimensional
5-FU	5-fluorouracil
ADC	apparent diffusion coefficient
AIC	Akaike information criteria
AIF	arterial input function
AUC	area under the curve
BF	blood flow
BIC	Bayesian information criteria
BTv	biological target volume
BV	blood volume
C	compartment (in pharmacokinetic modelling)
CI	confidence interval
CA19-9	carbohydrate antigen 19-9
CAIX	carbonic anhydrase IX
CECT	contrast-enhanced CT
cm	centimetre
cMR	complete metabolic response
CRM	circumferential resection margin
CRT	chemoradiotherapy
CT	computed tomography
CTV	clinical target volume
Cu-ATSM	⁶⁰ Cu-diacetyl-bis (N4-methylthiosemicarbazone)
DAB	diaminobenzidine
DFS	disease-free survival
dce-MRI	dynamic-contrast-enhanced magnetic resonance imaging

dw-MRI	diffusion-weighted magnetic resonance imaging
FAZA	^{18}F -fluoroazomycinarabinofuranoside
FDG	^{18}F -fluorodeoxyglucose
FLT	^{18}F -fluorothymidine
FMISO	^{18}F -fluoromisonidazole
FOV	field of view
GLUT	glucose transporter
GBSM	gradient-based segmentation method
GTV	gross tumour volume
Gy	Gray
h	hour
H&E	haematoxylin and eosin
HNSCC	head and neck squamous cell cancer
HIF-1 α	hypoxia-inducible factor-1 α
HU	Hounsfield unit
IHC	immunohistochemistry
IMRT	intensity-modulated radiotherapy
K	parameter in pharmacokinetic modelling
kg	kilogram
kV	kilovolt
LAPC	locally advanced pancreatic cancer
LARC	locally advanced rectal cancer
LD	local disease
mA	milliampere
MBq	megabecquarels
MC	motion corrected
mCR	metabolic complete response
MD	metastatic disease
MDT	multidisciplinary team
MET	^{11}C -methyl-L-methionine
mg	milligram
mL	millilitre

mm	millimetre
mmol	millimole
MRF	Markov random field
MRI	magnetic resonance imaging
mrTRG	MRI tumour regression grade
mTOR	mammalian target of rapamycin
ng	nanogram
NPV	negative predictive value
NSCLC	non-small cell lung cancer
OCIC	Oxford Cancer Imaging Centre
OPN	osteopontin
OS	overall survival
pAKT	phosphorylated Akt
PCA	principal components analysis
pCR	pathological complete response
pCT	perfusion computed tomography
PET	positron emission tomography
PFS	progression-free survival
PI3K	phosphatidylinositol 3-kinase
PPV	positive predictive value
PTV	planning target volume
PVC	partial volume correction
PVE	partial volume effects
RFS	recurrence-free survival
RNA	ribonucleic acid
ROC	receiver operator characteristic
ROI	region of interest
s	second
SUV	standardised uptake value
TAC	time activity curve
T:B	tumour to blood ratio
TLG	total lesion glycolysis

T:M	tumour to muscle ratio
TME	total mesorectal excision
TPZ	tirapazamine
TRG	tumour regression grade
TV	tumour volume
U	unit
UICC	Union for International Cancer Control

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Chapter 1: Introduction

Treatment strategy selection in cancer therapy is, for the most part, guided by tumour staging based on anatomical, cross-sectional imaging. Cancer staging, using the Union for International Cancer Control (UICC) TNM staging system describes local extension of the tumour (T), lymph node involvement (N) and whether or not the tumour has metastasised to distant sites (M). The term 'locally advanced' is commonly used to describe a tumour and any involved local lymph nodes that cannot be successfully surgically removed because of proximity to or invasion into unresectable normal structures in the absence of metastatic disease. This patient group is often treated with a combination of radiotherapy and chemotherapy (chemoradiotherapy, CRT). The aim of CRT in the locally advanced tumour setting is either to downstage the tumour to improve the chances of successful surgical resection (neoadjuvant CRT) or as a definitive, radical treatment that aims to achieve lasting control of tumour growth. The use of CRT has improved long-term cancer survival rates across a variety of tumour types.¹⁻⁴

The work in this thesis investigates personalising CRT strategies in two tumour sites: locally advanced pancreatic cancer (LAPC) and locally advanced rectal cancer (LARC). The natural history of LAPC and LARC and the aims of CRT, and response to CRT in these tumour types, are very different. The priorities and aims of any modifications to the investigation and treatment of patients with these cancer types are therefore also different. Pancreatic cancer has a very poor outlook, with only 1% of people diagnosed with pancreatic cancer achieving long-term survival. There has been no improvement in pancreatic cancer survival statistics in the past decade.⁵ In contrast, neoadjuvant CRT followed by surgical resection can achieve long-term control in 75% of cases of LARC.^{4,6}

Response to CRT varies between patients. Patients with the same tumour type and stage on anatomical imaging can have markedly different responses to treatment. While some patients are cured with CRT, others experience local or distant recurrence at a later date, and in some the cancer progresses during the treatment course. New treatment paradigms are emerging as knowledge of the biology of LAPC and LARC evolves. Accurate biomarkers are required to enable appropriate patient selection for differing approaches to treatment modification.

Cancer imaging does not have to be limited to providing only anatomical, staging information. Functional imaging allows a non-invasive assessment of physiological properties within tumours. This allows heterogeneity in tumour biology between patients with the same tumour type to be characterised. Physiological processes that can currently be characterised with imaging techniques include glucose metabolism, cell proliferation, hypoxia, perfusion, water diffusion and cell surface receptor expression characteristics. ^{18}F -fluorodeoxyglucose (FDG) positron emission tomography (PET) informs about glucose uptake and retention and is by far the most commonly used functional imaging test. Image-derived parameters can be used in risk stratification by quantifying biological features, detecting response to therapy, as well as providing information on intratumoural heterogeneity. An example of how tumour biology changes as a result of CRT, in this instance a complete metabolic response as seen on FDG-PET, can be seen in Figure 1.

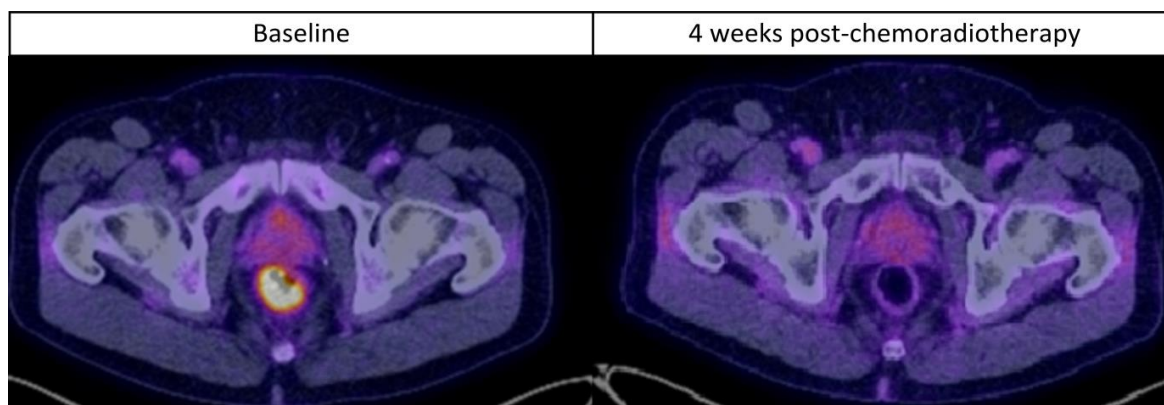


Figure 1: FDG-PET/CT demonstrating a complete metabolic response post CRT in a patient with a lower rectal adenocarcinoma (T3 N1 M0).

Radiotherapy is currently planned using computed tomography (CT). A planning CT is taken and the target for radiotherapy is delineated on the CT image. Further anatomical and functional imaging may be used as a reference, with or without registration of the image with the planning CT, to aid target volume definition at the discretion of the treating clinician. The first step is to delineate the gross tumour volume (GTV), defined as any macroscopic disease. Completion of the target volume for treatment requires expansion of the GTV to take into account microscopic tumour infiltration (the clinical target volume, CTV). A margin to account for tumour and patient motion and inaccuracies in treatment delivery is then added to produce the planning target volume (PTV). A radiotherapy plan that typically aims to deliver a homogenous dose to the PTV is then generated. The prescribed delivered dose to the PTV is decided from clinical trials and is not routinely altered on an individual patient basis. The treatment is then delivered with an assessment of response some time after completion of therapy. This standard treatment planning and delivery process is summarised in Figure 2(A). There are a number of ways in which CRT for LAPC and LARC could be personalised using functional imaging. In Figure 2(B) it is proposed that a pre-treatment functional imaging assessment of a tumour characteristic could be used to personalise therapy, either in helping delineate a target volume or in adapting the

dose prescription or chemotherapy based on response prediction using imaging-derived biomarkers. Alternatively, as depicted in Figure 2(C), assessing response by repeating a functional imaging test early in a course of CRT may be used to modify the remainder of the treatment. Before either of these strategies is investigated further, a greater understanding of how imaging-derived parameters correlate with individual patient response to therapy is required.

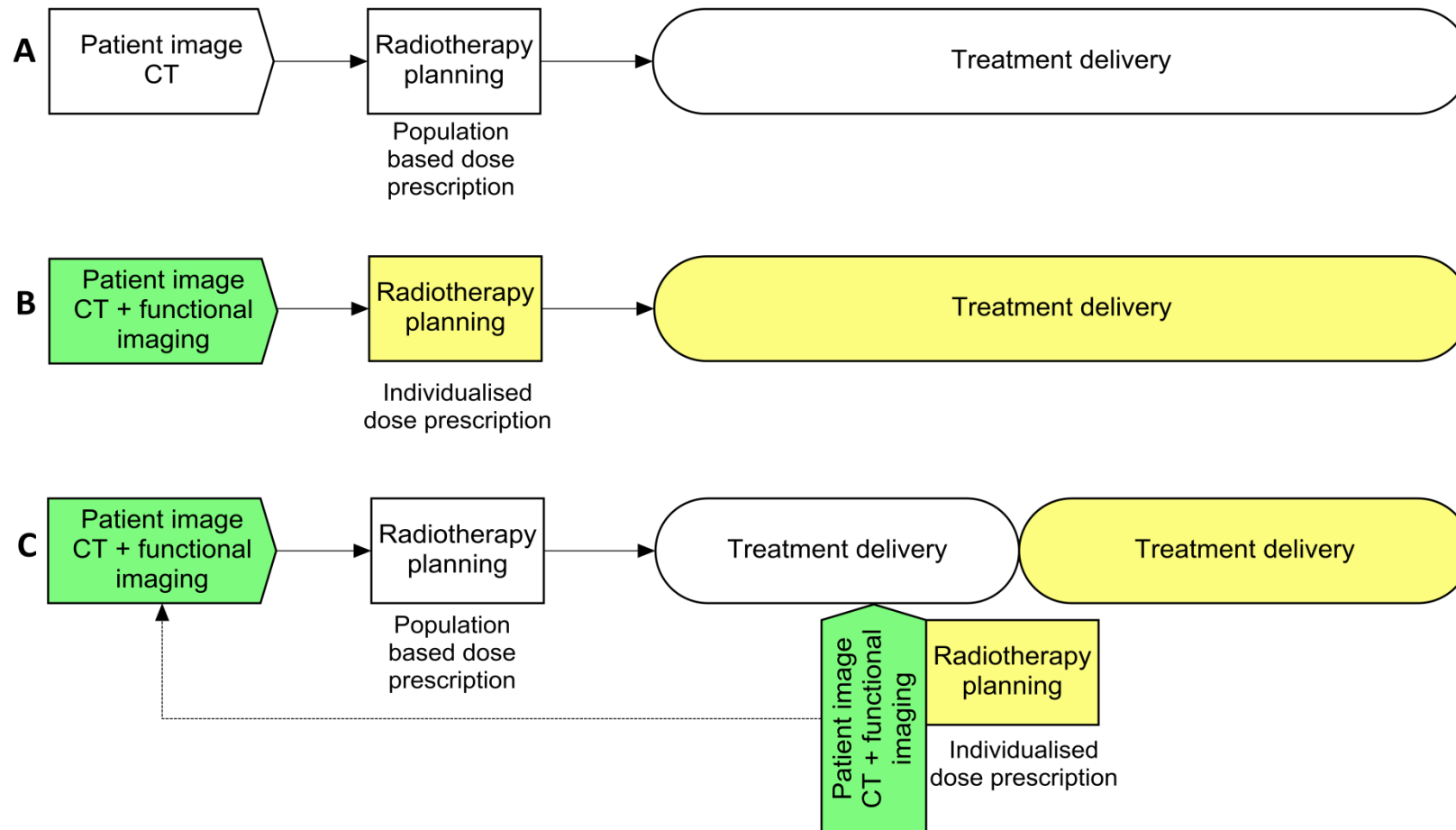


Figure 2: Integrating functional imaging into CRT strategies. (A) Depicts the current method of planning radiotherapy planning. A target volume is delineated on a CT image and a population-based radiotherapy dose is delivered. (B) A functional assessment of the tumour may predict tumour radiosensitivity and help delineate an accurate target volume, allowing the delivery of an individualised CRT prescription. (C) The patient starts a population-based CRT strategy, but response to treatment is assessed early in the treatment course allowing for adaption of the remaining treatment, by changing the radiotherapy or chemotherapy, to optimise treatment outcomes.

Standards of radiotherapy planning, prescribing and planning are set, by international consensus, by the International Commission on Radiation Units & Measurements. An emphasis on delivering a homogenous dose distribution to the PTV has been at the centre of standard radiotherapy practice. An example of an intensity-modulated radiotherapy (IMRT) plan for LARC can be seen in Figure 3. While it is possible to deliver a homogenous dose, with increasing degrees of conformality using modern radiotherapy techniques such as the IMRT used here, often large volumes of tissue are bathed with a uniformly high dose. This can have consequences for the patient in terms of acute and late-occurring normal tissue toxicity.

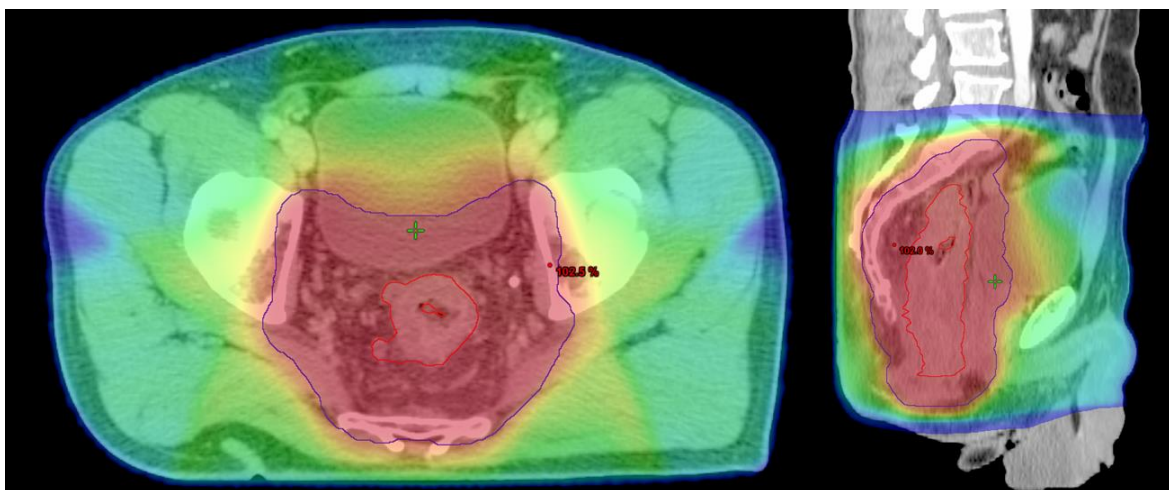


Figure 3: Axial and sagittal sections from a 7-field IMRT plan for LARC. The GTV (red line) and PTV (blue line) can be seen.

Ensuring radiotherapy delivery platforms deliver a uniform dose to the PTV at all times assumes that the radiosensitivity of the tumour is homogenous throughout its volume. Areas that are 'at risk' rather than being definitely involved by tumour may also be safe to be treated to a lower radiotherapy dose. As can be seen in Figure 3, treating the entire PTV to a homogenous dose often leads to treating large volumes, including significant volumes of normal tissues, to a considerable dose.

Dose painting aims to deliver a deliberately non-uniform dose distribution across the PTV. This requires the accurate characterisation of intratumoural heterogeneity and the localisation of radioresistant regions for selective dose escalation or radiosensitive areas for radiotherapy dose reduction. A distinction has been made between dose painting by volume, which requires the delineation of a biological target volume (BTV), and dose painting by numbers, which varies the dose on a voxel-by-voxel basis within the tumour volume (TV).⁷ It is likely that dose painting will ultimately be limited by the spatial resolution of the imaging modality used and the length-scale over which it is possible to modulate the delivered dose. Tumour heterogeneity in terms of radiosensitivity is likely to vary on a micrometre-by-micrometre scale. The spatial resolution of most functional imaging studies is in the order of millimetres. Some assumptions of radiosensitivity, with averaging out of signal intensity across the image voxel, are therefore inevitable. A schematic representation of how a traditional uniform (A) and a non-uniform distribution that varies the radiation dose across the tumour using data about tumour biology derived from a PET image (B) could be delivered in a clinical trial, in the example given in head and neck squamous cell cancer (HNSCC), can be seen in Figure 4.

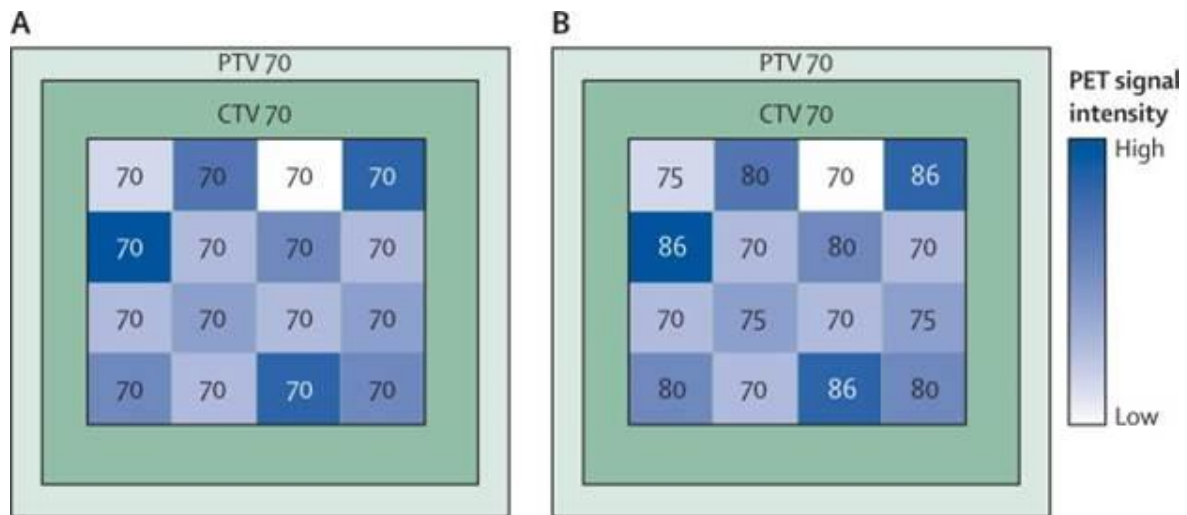


Figure 4: A schematic representation of a uniform radiotherapy dose distribution (delivering 70 Gy to the entire PTV and CTV) while keeping the dose uniform across the GTV (A) or dose painting by numbers based on PET signal intensity within the GTV (B). (Reproduced, with permission, from Gregoire *et al.*⁷⁾

Before this approach can be widely adopted across tumour sites, the relationship between the physiological process being characterised in the functional imaging study with radiosensitivity and treatment outcome would have to be established. There would also need to be confidence in the test-retest repeatability of the quantitative data and geographical distribution and stability of the imaging signal in the functional imaging test.

Tumour hypoxia has been known to lead to radioresistance since 1953.⁸ Hypoxia arises in tumours because of an increased cellular consumption of oxygen and reduced delivery of oxygen because of the highly abnormal tumour microvasculature. Poor vascularity characterises more than half of human tumours regardless of histology and grade.⁹ There is an intrinsic dependence of radiotherapy on cellular oxygen to cause lethal damage to the tumour cell. While cell kill is possible in the absence of oxygen, it is greatly enhanced by its presence. In clinical studies, higher rates of local and distant failure following chemotherapy and radiotherapy are seen in hypoxic tumours.¹⁰⁻¹² The relationship

between hypoxia, chemo- and radiotherapy resistance and a more aggressive cancer cell phenotype in terms of cell survival, invasion and metastasis is a rapidly developing research priority in oncology. A comprehensive review of this literature is beyond the scope of this thesis, but has been reviewed elsewhere.¹³⁻¹⁶ The way in which we can quantify tumour hypoxia clinically and the need for a robust, non-invasive means of characterising tumour hypoxia will be discussed here.

The remainder of this introductory chapter will:

- Outline the current best practice in managing LAPC and LARC with CRT
- Describe specific ways in which biomarkers, image-derived or otherwise, might be used to personalise CRT in LAPC and LARC
- Discuss the findings of a systematic review that was conducted of the published literature concerning LAPC or LAPC and functional imaging and treatment response prediction and prognosis and BTV definition. Key themes and common limitations of these studies will be highlighted and the work in this thesis that aims to address some of the identified gaps in knowledge will be introduced
- Explore the significance of tumour hypoxia in clinical studies and the challenges in characterising hypoxia in human malignancies
- Review the most commonly used hypoxia imaging modality, ¹⁸F-fluoromisonidazole (FMISO) PET, literature with specific attention to tracer and methodological development, response prediction using FMISO-PET and the use of FMISO-PET to delineate hypoxic BTVs
- Introduce the original work presented in the remainder of this thesis

1.1 Pancreatic cancer

Pancreatic cancer is the tenth most common cancer in the UK and the fifth most common cause of cancer death. Around 8,700 people die from pancreatic cancer in the UK each year. Long-term survival following a diagnosis of pancreatic cancer is achieved in only 1% of patients, with no improvement in survival statistics seen over the past decade.¹⁷ Approximately 30% of pancreatic cancers are locally advanced, and therefore unresectable despite the absence of metastatic disease, at presentation.¹⁸ LAPC is treated by chemotherapy alone, upfront CRT, or induction chemotherapy followed by CRT. The median survival of patients treated with induction chemotherapy followed by CRT is approximately 15 months.^{19,20} In a multicentre, Phase III study, the addition of CRT following induction chemotherapy did not confer a survival advantage over the continuation of chemotherapy alone.²⁰ A focused local treatment might have been expected to improve patient outcomes as locoregional failure is the first site of disease progression in 25–29% of those with LAPC,^{21,22} and 28% of patients presenting with LAPC have no evidence of metastatic disease at autopsy.²³ This further supports the need for strategies that improve local control in a subset of patients. Conversely, an Eastern Cooperative Oncology Group study that randomised patients with LAPC to gemcitabine alone or in combination with radiotherapy (50.4 Gy in 28 fractions) revealed a small but statistically significant improvement in overall survival (OS) with CRT compared with gemcitabine alone (11.1 vs 9.5 months, $p = 0.017$), which supports the ongoing investigation of CRT strategies in this patient group.²⁴ It is also possible that an improvement in survival rate in all patients with LAPC would be seen by improving tumour response to CRT. In a series of patients with operable pancreatic cancer receiving neoadjuvant CRT, those with <10% of viable tumour cells post CRT had a median OS of 39

months compared with only 15 months in those who have >10% of viable tumour cells remaining.²⁵ This study not only identifies heterogeneity in response to CRT in pancreatic tumours, but also that a poor response is associated with a poor outcome. While failure to respond to CRT may simply be a marker of unfavourable tumour biology and therefore poor prognosis, the impact of strategies that increase tumour response to CRT on survival warrants further investigation.

An ability to predict which patients will have a locally progressive pattern of disease may identify a subgroup that might benefit most from CRT over chemotherapy alone. Loss of the tumour suppressor gene Smad4 (dpc4) is associated with the development of metastatic disease in pancreatic cancer.^{21,23} While stratification of patients based on Smad4 (dpc4) is included in the currently recruiting RTOG 1201 study, the diagnosis of pancreatic cancer is often made on scanty biopsy material or cytology, which limits the accuracy of SMAD4 immunohistochemistry.²¹ A non-invasive method of predicting the pattern of disease progression *a priori* may aid treatment strategy selection on an individual patient basis.

Carbohydrate antigen 19-9 (CA19-9) is well recognised as a tumour marker in pancreatic cancer.²⁶ A prognostic role for CA19-9 levels in LAPC is emerging. In a prospective series of 38 patients, low pre-treatment circulating levels of CA19-9 have been associated with prolonged OS and progression-free survival (PFS) following definitive chemoradiotherapy for LAPC.²⁷ In a retrospective analysis of 54 patients with LAPC treated with CRT, it was the relative change in CA19-9 from pre- to post CRT that was associated with a longer median OS (16.3 vs 7.5 months in those with a >90% or <90% decrease in CA19-9 respectively, $p = 0.02$).²⁸ The best time to quantify CA19-9 for it to most reliably predict patient outcome in

LAPC treated with CRT is not yet known. Levels of CA19-9 are not currently used to direct treatment strategy decisions.

1.2 Rectal cancer

More than 14,000 people were diagnosed with rectal cancer in the UK in 2011.²⁹ Currently, treatment decisions are directed by magnetic resonance imaging (MRI) appearances of the rectal tumour³⁰ once metastatic disease has been excluded by CT. Surgical resection is the cornerstone of treatment in the majority of cases. When the surgical circumferential resection margin (CRM) is involved or threatened (LARC), preoperative CRT is considered to be the current standard of care as it reduces the risk of local recurrence.^{4,31,32} Patients who achieve pathological complete response (pCR), no evidence of viable tumour in the resected specimen, following CRT have a better long-term treatment outcome than those who do not.³³ This may be a reflection of favourable tumour biology, but could also suggest that improving rates of pCR following neoadjuvant CRT may be of clinical benefit. With current standard neoadjuvant CRT regimens for LARC, fewer than 30% of patients achieve pCR.^{32,34-36} If rates of pCR could be increased, and pCR could be accurately identified on post-CRT imaging, the management of a subset of patients with LARC may eventually, as in anal cancer,¹ be definitively managed by CRT followed by active surveillance. This will spare patients the associated morbidity of total mesorectal excision (TME), which, with low tumours, requires abdominoperineal resection and is associated with considerable morbidity.³⁷ Accurate response prediction algorithms are required to identify those who are likely to achieve pCR following CRT and those who are not. Personalisation of CRT dose strategies may increase the rates of pCR in those with unfavourable tumour biology while avoiding normal tissue toxicity in those tumours that are responsive to CRT.

1.3 Personalising chemoradiotherapy strategies

Incorporating accurate biological stratification of tumours into diagnostic and surveillance investigations may help select patients for new treatment paradigms that target the underlying biology with the aim of ensuring sustained treatment response. In both LAPC and LARC, accurate identification of patients at high risk of a poor treatment response may justify treatment intensification strategies that improve local control rates at the expense of increased normal tissue toxicity. Conversely, in those patients with tumours that have favourable biological characteristics, a more conservative treatment strategy may be considered.

In the subgroup of patients with LAPC who only ever experience local tumour progression in the absence of metastatic spread,²¹⁻²³ escalating the radiotherapy dose to the pancreas appears attractive. Previous attempts to dose escalate to the entire GTV have been limited by normal tissue toxicity, particularly to the duodenum.³⁸ *A priori* identification of patients in whom the disease burden will only be at the primary site, may identify a group of patients who would benefit from local treatment intensification. For example, patients with intact Smad4 (Dpc4) expression tend to have a local pattern of progression compared with those with Smad4 (Dpc4) loss, who tend to have a distant pattern of progression.²¹ Pancreatic tumour biopsy is challenging and does not always provide enough material for detailed analyses such as Dpc4 expression. It also does not inform about intratumoural heterogeneity and could not be used to identify 'high risk' areas of the GTV for radiotherapy dose escalation.

In LARC, current CRT adopts an approach where a large volume within the pelvis is bathed in a uniform dose, as seen in Figure 3. Personalised modification of neoadjuvant CRT for

LARC may increase the number of patients who can be optimally managed with organ-preserving strategies, making CRT the definitive treatment. The utility of conventional anatomical imaging, such as MRI-derived changes, in differentiating those with pCR post CRT is uncertain. While some series have failed to show a correlation between MRI-volumetric change with rates of pCR³⁹ or tumour regression grade (TRG),⁴⁰ a qualitative assessment of post-CRT MRI appears to identify those who have failed to respond with a degree of accuracy, but performs poorly in predicting a good pathological response.⁴¹ A means of risk-stratifying tumours pre-CRT to guide strategy selection and post CRT to accurately assess response if a non-surgical, active surveillance approach is to be adopted is needed in LARC.

There is growing support for the adoption of active surveillance rather than surgical resection in those with a clinical and radiological complete response following CRT, with some evidence that long-term outcomes in this group are as good as those managed by TME.^{33,42,43} This approach requires a confidence in the ability of post-CRT imaging to accurately identify pCR.

In both LAPC and LARC, identification of imaging biomarkers that accurately risk-stratify patients and reliably identify a subvolume within the tumour that is associated with chemoradioresistance may guide treatment selection and allow for selective radiotherapy dose escalation to a BTV. Deliberately delivering an inhomogeneous radiotherapy dose to the pancreatic or rectal TV, allowing for higher dose deposition within the BTV, may spare normal tissues. The confident definition of a BTV may also permit dose reduction to low-risk areas within the pancreatic GTV or pelvis.

1.4 Functional imaging

Functional imaging is a variety of clinical imaging modalities to allow a non-invasive assessment of the heterogeneity of physiological properties within individual tumours and between patients with the same tumour type. Image-derived parameters can be used in risk stratification by quantifying biological features, detecting response to therapy, as well as providing information on intratumoural heterogeneity.

FDG-PET is increasingly used for accurate tumour staging. The accurate detection or exclusion of metastatic disease is essential before embarking on, or excluding a patient from, radical therapy. In a series of 338 patients who were thought to have LAPC on anatomical imaging, 33% were found to have metastatic disease when a staging FDG-PET/CT was added to the diagnostic pathway.⁴⁴ In those who did not have metastatic disease, 6.5% of patients had FDG-avid malignant lymph nodes that had not been identified on standard imaging. An example of FDG-PET revealing previously unrecognised local lymph node involvement can be seen in Figure 5. FDG-PET alters the TNM stage of patients with rectal cancer in approximately 30% of patients.⁴⁵ In 12% of cases, previously unrecognised lymph nodes were identified by FDG-PET,⁴⁶ which would have implications for GTV definition during radiotherapy planning.

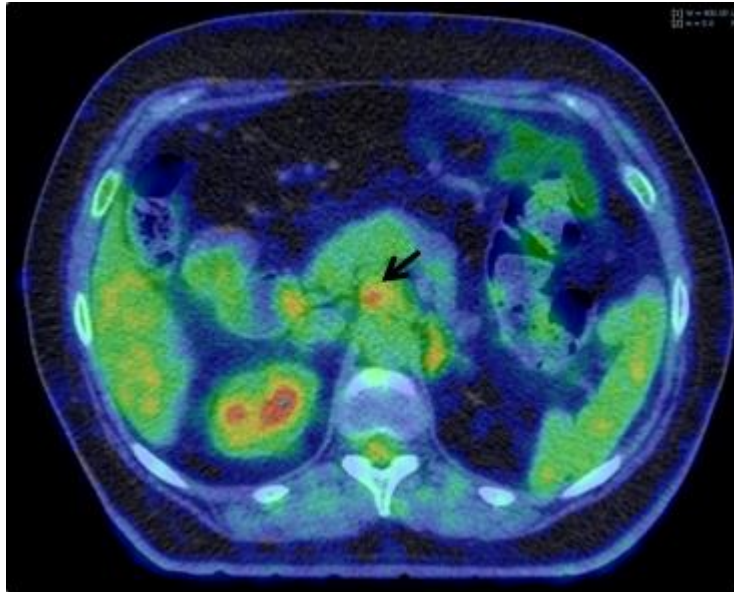


Figure 5: Axial image from an FDG-PET/CT in a patient with LAPC. An FDG-avid peripancreatic node that was not identified at the time of diagnostic contrast-enhanced CT was detected and included in GTV definition at the time of radiotherapy planning.

Functional imaging has therefore been shown to improve the accuracy of cancer staging, but its application into individualising CRT treatment paradigms has not yet been adopted in the treatment of LAPC or LARC.

1.5 Integrating functional imaging into treatment strategy selection and radiotherapy planning

Differences in imaging-derived parameters at baseline and response of these parameters to treatment between patients may provide a means of risk stratification of individual patients that could then be used to guide treatment decisions. If treatment modification based on information obtained from functional imaging is to be considered, a robust prediction of outcome would need to be available before, or early in the CRT course. An imaging-derived biomarker could be prognostic, predictive, or both. Prognostic biomarkers provide information about long-term patient outcomes such as survival or time to local progression. Predictive biomarkers identify patients who are likely to respond to a

treatment. The end-point of studies looking at functional imaging in LAPC is more likely to investigate the prognostic utility of the test as it is unusual to achieve objective response rates on anatomical imaging post CRT for LAPC, and few patients go on to have surgical resection post CRT. Studies in LARC are likely to derive predictive information from functional imaging tests. As CRT is given as neoadjuvant therapy in LARC, an objective response assessment is possible, mostly notable histopathological TRG. Studies that have used functional imaging in patients receiving CRT can therefore be predictive or prognostic.

A systematic review of the literature to characterise the predictive and prognostic utility of functional imaging in LAPC and LARC beyond that offered by accurate staging was performed. A second search investigating the application of functional imaging to radiotherapy target volume definition in both LAPC and LARC was completed. These searches were performed to inform discussion as to how functional imaging may inform proposed adaptation of the current CRT strategies. Details of the literature search strategies can be found in Appendix A.

All studies investigating the predictive and prognostic utility of functional imaging in LAPC used FDG-PET/CT. A summary of these trials is provided in Table I of Appendix A. The number of studies in pancreatic cancer was considerably lower than the number in rectal cancer – the database search identified 66 studies concerning functional imaging as means of predicting CRT response, only 7 of which were eligible for inclusion after full text review. Only one study using functional imaging to guide target volume definition in pancreatic cancer was identified by this search strategy. This study also employed FDG-PET/CT.

A number of PET imaging-derived parameters have been used to predict treatment outcome. For PET images, the standardised uptake value (SUV), which is the measured

activity within an image voxel corrected for time, injected activity and body weight, is most commonly used. This can be represented using the maximum SUV (SUV_{max}), mean SUV across a region of interest (SUV_{mean}), or in the case of FDG-PET combined with a measure of TV to produce the total lesion glycolysis (TLG). The apparent diffusion coefficient (ADC), a quantification of the restrictions to diffusion of molecules by factors such as differing cellular density, is derived from diffusion-weighted MRI (dw-MRI). Parameters such as blood flow, mean transit time and blood volume from perfusion CT (pCT) and K^{trans} from dynamic contrast-enhanced MRI (dce-MRI) are derived from dynamic compartmental models.

Patient outcomes following CRT for LAPC are varied. Few studies have investigated the predictive or prognostic utility of functional imaging in LAPC. In one large series of 260 patients, patients with either a baseline FDG-PET $SUV_{max} < 3.5$ or a decrease in SUV_{max} ($\% \Delta SUV_{max}$) $\geq 60\%$ from pre- to post CRT had better OS and PFS than those who did not.⁴⁴ This study was limited by a heterogeneity of CRT regimens within the cohort studied and considerable variation in the timing of FDG-PET in relation to the CRT. The universal applicability of these parameters as prognostic biomarkers was examined. The results of this testing are presented in Chapter 2.

A summary of all of the reported trials using functional imaging as a predictive or prognostic biomarker in rectal cancer can be seen in Tables II–V in Appendix A. Studies are grouped according to the timing of the imaging in the CRT schedule – either before CRT alone (7 studies) or pre-CRT and during CRT (19 studies), pre- and post CRT (38 studies) or post CRT alone (6 studies). Forty-three studies used FDG-PET for response prediction. The other imaging modalities employed in the studies were dw-MRI (11 studies), dce-MRI (7

studies), pCT (3 studies), ¹⁸F-fluorothymidine (FLT) PET (2 studies) and ¹¹C-methyl-L-methionine (MET) PET (1 study).

While the magnitude of a parameter, or the direction of change in the parameter, used to predict treatment outcome is usually the same across studies, this is not the case for ADC in LARC. The mean pre-CRT ADC has been demonstrated to be higher⁴⁷ or lower⁴⁸ in responders compared with non-responders in different studies.

FLT uptake is a marker of cellular-proliferation and FLT-PET has been used in LARC. FLT-PET was able to demonstrate a reduction in tracer uptake from baseline to the end of the second week of CRT and post CRT, but no difference in FLT-PET-derived parameters was seen between responders and non-responders.^{49,50} MET-PET has also been used in LARC, but parameters were no different between responders and non-responders⁵¹. FLT- and MET-PET will therefore not form part of the following discussion.

In LARC, the end-point to be predicted varied between studies. Twenty-two series used functional imaging to predict pCR (no evidence of tumour within the resected specimen). Studies were more likely to characterise residual disease using TRG, such as the method described by Mandard *et al.*⁵² or Dworak *et al.*⁵³ TRGs are histopathological scoring systems that categorise the response to cytotoxic therapies, ranging from progression during treatment to complete response. The Mandard and Dworak TRG systems are summarised in Table 1. When TRG was used as the histopathological end-point to be predicted, many studies labelled individuals with Mandard TRG 1 and 2 (complete regression or rare residual tumour cells) or Dworak TRG 3 and 4 (no or very few residual tumour cells) as 'responders'.

Table 1: Tumour regression grading systems as described by Mandard *et al.* and Dworak *et al.*

Mandard TRG	Dworak TRG
1. Complete regression (fibrosis, absence of tumour cells)	1. No regression
2. Rare tumour cells scattered through fibrosis	2. Dominant tumour mass with obvious fibrosis and/or vasculopathy
3. Fibrosis and tumour cells with a preponderance of fibrosis	3. Dominantly fibrotic changes with few tumour cells or groups (easy to find)
4. Residual cancer outgrowing fibrosis	4. Very few (difficult to find microscopically) tumour cells in fibrotic tissue with or without mucous substance
5. Absence of regressive changes	5. No tumour cells, only fibrotic mass (total regression or response)

Functional imaging studies in LARC that aimed to inform about prognosis following CRT and TME are summarised in Table V of Appendix A. Again, trials are grouped into those that performed imaging pre-CRT alone (4 studies), pre- and during CRT (2 studies), pre- and post CRT (9 studies) or post-CRT imaging only (2 studies). Fifteen of these studies used FDG-PET, while dw-MRI and ^{60}Cu -diacetyl-bis (N4-methylthiosemicarbazone) (Cu-ATSM) were each used in one study.

All of the studies looking at radiotherapy target volume definition in LARC used PET. Nine utilised FDG-PET and one study discussed the incorporation of FDG-, FMISO- and FLT-PET into BTV definition. Of note, the interobserver variability in GTV definition, as assessed by the coefficient of variation and a concordance index, was reduced by inclusion of FDG-PET into the planning process.⁵⁴

Functional imaging is not yet used routinely in clinical practice to risk-stratify individual tumours or to derive BTVs in either LAPC or LARC. A number of common themes can be seen in the literature that may explain why functional imaging is yet to emerge as a means of personalising CRT for universal clinical application.

1.5.1 Treatment strategy selection

A number of common trends emerge from the methodology of functional imaging studies in both LAPC and LARC that make finding an image-derived parameter that will accurately identify those patients who are unlikely to achieve a favourable response to CRT, or poorer OS, and possibly benefit from dose intensification less likely. These are:

1.5.1.1 Selection of a treatment outcome end-point for response prediction

Only one study that investigated the predictive power of FDG-PET in pancreatic cancer was identified.⁵⁵ This series was in patients with T3 tumours (cancers that extend beyond the pancreas, but do not involve the coeliac axis or superior mesenteric artery), resectable pancreatic cancer, and CRT was given as a neoadjuvant treatment. This series defined histopathological response as <50% viable tumour cells seen in the resection specimen. This end-point was perhaps chosen because only 2% of patients achieved pCR post CRT. The impact that achieving a reduction to <50% of viable tumour cells has on patient outcome is not yet understood, so the value of predicting this end-point is uncertain.

In LARC, some studies used tumour down-staging, in terms of local tumour extension, seen at histopathological assessment to define a favourable response.⁵⁶ It is known that MRI can over-stage tumours, so when comparing with the histopathological ground truth an artificial inflation in the number of 'responders' may be observed.⁵⁷ More studies used a favourable TRG as a good response than used pCR. While patients with Mandard TRG 1–2

have a better prognosis than those with TRG 3–5 in terms of local failure, metastases-free survival and OS,⁵⁸ this end-point is a good first step towards patient selection, but it will not identify those in whom a non-operative approach may be considered. Confidence in imaging studies to accurately identify pCR (Mandard TRG 1 or Dworak TRG 4) is needed before imaging studies alone can be used to identify patients with rectal cancer who can be offered organ-sparing, non-operative management following CRT. Treatment personalisation that relies on functional imaging to inform treatment strategy decisions such as radiotherapy or chemotherapy intensification or reduction should therefore aim at optimising rates of pCR.

1.5.1.2 Parameter cut-offs in response prediction

Most studies rely on analysis of the receiver operator characteristic (ROC) curve to define a parameter cut-off to separate responders from non-responders. The sensitivity of a test is the proportion of patients with a specified outcome that will be correctly identified by the test. For example, if all of the patients who had a pCR following CRT had a reduction in the $SUV_{max} > 50\%$, the sensitivity of the test (reduction in $SUV_{max} > 50\%$) is 100%, or 1. The specificity of a test concerns the number of patients for whom the outcome of the test is negative who are correctly identified. For example, if all of the patients who did not have a reduction in the $SUV_{max} > 50\%$ did not have a pCR, the specificity would be 100%, or 1. (1 – specificity) therefore gives an indication of the number of false negative test results. The ROC curve plots the sensitivity of a test against (1 – specificity) of the test. As a perfect test would have a sensitivity and specificity equal to 1, the ROC curve would start at (0,0), travel vertically along the y-axis to (0,1) and then across to (1,1). The area under the curve (AUC) of this ROC curve would therefore be 1.0, indicating the perfect test. In comparison, a

random guess of the outcome of the test would generate a ROC curve with an AUC of 0.5.

The ROC curve AUC is therefore a useful indicator of how accurate a diagnostic test is.

When the outcome of a test is a continuous variable (e.g. change in SUV_{max} from pre- to post CRT), the parameter threshold can be selected to optimise the sensitivity or specificity of the test, or to compromise and optimise both the sensitivity and specificity. The cut-off threshold chosen may not have any physiological basis or, in the case of a response index, may be within the inherent uncertainty in the imaging parameter between time-points. Accurate quantification of the test-retest repeatability of the parameter is required so the magnitude of any response that should be considered to be significant can be established. For example, if the intrinsic uncertainty of an imaging-derived parameter is in the order of 20%, it would not make sense to use a parameter change threshold of less than 20% in a response prediction algorithm. In addition, the parameter cut-off that is often chosen appears to have been selected to optimise the sensitivity of the test or as a compromise to maximise both sensitivity and specificity. If the aim of a treatment intervention is to improve rates of pCR, perhaps with the hope of organ preservation, a greater importance should be given to improving the *specificity* of the test to accurately identify those who are failing to have an optimal response early to allow for treatment intervention. Wrongly ascribing a patient to the 'responder' category rather than identifying their failure to respond could have catastrophic consequences for the patient. Specificity of 100% would correctly identify those who are failing to respond, and allow dose-intensification for the remainder of the treatment, but could falsely identify a number of patients who were likely to achieve pCR without intervention as 'non-responders'. This potential treatment

intensification to a proportion of patients who would not derive any personal benefit must be taken into account when deciding if a proposed trial intervention is acceptable or not.

1.5.1.3 Image interpretation

The predictive value of post-CRT FDG-PET in predicting pCR post neoadjuvant CRT for LARC is often low. The accuracy was only 23% in one large series of 151 patients.⁵⁹ This is often attributed to FDG avidity as a result of inflammation, oedema and fibrotic reaction post CRT rather than FDG avidity representing viable tumour activity. Quantitative analysis of FDG-PET images can never be fully automated as a region of interest (ROI) needs to be defined. Some qualitative assessment of the imaging is therefore always necessary. Attempts to standardise these imaging assessments as CRT FDG-PET response criteria such as the Hopkins criteria, which differentiates between focal and diffuse FDG uptake, have been shown to have good interobserver reliability and prognostic function in HNSCC⁶⁰ and may be useful in both pancreatic and rectal cancer. When dw-MRI was applied in LARC, objective, reproducible, quantitative parameters were essential in using imaging in risk stratification. When a qualitative assessment of dw-MRI was performed by two independent radiologists, ROC curve AUC was 0.676 and 0.658 for predicting pCR; this improved to 0.925 when a quantitative assessment using a threshold of ADC was used.⁶¹ This highlights the need for a qualitative analysis of the image before using automated or semi-automated methods to extract image-derived parameter values.

Some studies looking at dce-MRI and dw-MRI in LARC used a single axial slice through the tumour, assuming it to be representative of the whole TV.⁶² It has been demonstrated that ROI size and positioning have significant effect on ADC values, but the most reproducible results are obtained when the entire TV is taken into account rather than single slices or

fixed volume ROIs.⁶³ Many studies calculated ADC on a single slice and others used smaller ROIs with a fixed shape.⁶⁴ ROI definition should be standardised to allow results from different series to be accurately compared and to reduce any potential bias in ROI placement for parameter quantification. Delineation of ROIs is, however, subject to inter- and intraobserver variability.

1.5.1.4 Dependence on FDG-PET SUV_{max}

Future studies that use PET may be improved by not relying heavily on the SUV_{max} . The SUV_{max} is a single-pixel value within a region of interest and is subject to considerable noise.⁶⁵ When looking for a change in SUV_{max} , particularly post CRT when uptake volumes may be small, partial volume effects (PVE) should also be taken into account. The spatial resolution of PET is limited and volumes smaller than approximately 2.5 times the full-width at half-maximum will have an artificially reduced SUV_{max} unless a partial volume correction (PVC) is applied.⁶⁶ Only one study, in LARC, assessed the impact of PVC on the predictive utility of FDG-PET parameters on images taken pre-CRT and two weeks into CRT.⁶⁷ PVC, in this instance, did not significantly increase the ROC curve AUC. The impact of PVC on other series, particularly those with post-CRT response indices, where the volume of residual tracer avidity is likely to be smaller, is not known. The impact of PVC on FDG-PET-derived parameter values in LAPC is also not yet known.

Use of relative change in SUV_{mean} from baseline to day 12 of CRT showed a higher ROC curve AUC than using SUV_{max} (0.95 vs 1.00).⁶⁸ The relative reduction in SUV_{mean} was also more reliable in predicting five-year recurrence-free survival (RFS) than relative reduction of SUV_{max} .⁶⁹ This may in part be due to the reduction in the uncertainty of the parameter by averaging SUVs over a ROI, and diminishing the impact of noise rather than relying on a

single point value. Extracting as much information as possible from the image may reduce uncertainty and improve predictive power. Rather than relying on a point value, a detailed analysis of heterogeneity of FDG uptake within a TV including texture features has been shown to increase the predictive accuracy of FDG-PET in oesophageal cancer.⁷⁰ This approach has not yet been tested in LAPC or LARC. A suggestion that use of kinetic parameters from dynamic imaging may improve the predictive utility of the test in LARC is supported by the observations of Yoon *et al.*⁷¹ They observed an ROC curve AUC of 0.703 using post-CRT SUV_{max} to predict TRG. Using the same imaging time-point, the ROC curve AUC improved to 0.906 if the relative change in SUV_{max} from an image acquired 60 min post injection to one acquired at 90 min was used to predict response. Further investigation of dynamic-PET in CRT response prediction in LAPC and LARC is warranted and may identify a parameter that has a higher predictive value than SUV_{max}.

1.5.1.5 Timing of functional imaging to aid patient response stratification

Relying on a post-CRT assessment of response does not allow intervention to intensify treatment in those failing to show a favourable response. In LARC, parameters derived from imaging taken at baseline failed to separate responders from non-responders in a number of series across imaging modalities (see Table III in Appendix A). The need for imaging the patient pre-CRT appears to be solely to allow a relative response assessment at a later time-point, which often offers predictive and prognostic information, even if the baseline imaging did not.⁷²⁻⁷⁴ When a number of FDG-PET-derived parameters were compared in the same cohort of patients, relative response quantification appeared to be a more robust means of predicting TRG (AUC 0.862) than absolute values at a single time-point (AUC 0.716) or absolute change (AUC 0.806).⁷⁵ A similar pattern was seen in LAPC.

All studies to assess the predictive and prognostic utility of functional imaging in pancreatic cancer used FDG-PET. Only one study that showed a correlation with FDG-PET parameters with treatment response was found. Higher baseline SUV_{max} was associated with histopathological response – the predictive function of FDG-PET in this series was increased by combining the baseline SUV_{max} with the relative SUV response post CRT.⁵⁵

Four studies looked for correlation between FDG-PET and patient prognosis in LAPC. An association with low baseline SUV_{max} and larger SUV_{max} reduction following CRT was observed.³³ All of the studies in LAPC used imaging pre- and post CRT. One retrospective series of patients who received a variety of radiotherapy-based treatments for LAPC identified that a low pre-CRT FDG-PET SUV_{max} and a reduction in SUV_{max} > 60% was associated with prolonged OS. Again, a dependence on post-CRT imaging that will not allow for early intervention in those failing to respond was observed.

Repeat imaging early in the course of CRT to help in risk stratification shows promise in LARC. FDG-PET imaging at day 15 is better at identifying those with LARC who are failing to respond than at day 8 (ROC curve AUC 0.79 vs 0.73).⁶⁷ Changes in dw-MRI as early as one week into CRT appear to separate those likely to have a favourable response from those who are not.⁷⁶ Early changes in dce-MRI did not differentiate patients who went on to have a favourable TRG from those who did not, but there was a difference in pre- and post-CRT K^{trans} in terms of tumour downstaging on anatomical imaging, but not histopathological response.⁷⁷

The timing of reported post-CRT imaging was highly variable in patients with LARC, ranging from 2–4 weeks⁷⁸⁻⁸⁰ to as long as 10 weeks after completing treatment.⁸¹ The time-point that offers the most accurate predictive information is currently unclear, although it has

been suggested that using FDG-PET before 4 weeks increases the likelihood of a false-positive result because of radiotherapy-associated inflammation leading to a false-positive FDG-PET.⁸²

1.5.1.6 Specific histological subtypes of rectal cancer

Mucinous rectal tumours are difficult to interpret on conventional MRI post CRT as it is difficult to differentiate mucin pools from residual tumour. This is not particularly improved by dw-MRI, although they do tend to have higher ADCs than other adenocarcinomas.⁶¹ Parameters derived from dce-MRI are significantly different between mucinous and non-mucinous rectal cancers.⁸³ This suggests that different parameter thresholds may be required to risk-stratify tumours that have a predominantly mucinous phenotype. Most studies do not differentiate between histological subtypes, making extrapolation of the derived parameter values used to predict treatment response to an individual patient more challenging.

1.5.1.7 Need for validation

Most of the studies were conducted in a single institution, with no external validation of the predictive parameter thresholds. One series of patients with LARC, identified that a reduction in SUV_{max} from baseline to after the second week of CRT identified responders with a specificity of 100% (ROC curve AUC 0.980).⁸⁴ They then applied this threshold to a separate cohort of 20 patients. When applied to this group, the sensitivity and specificity were 83% and 93% respectively. All parameters that are to be taken forward in a trial to individualise CRT require external validation. This will require cross-centre verification and quality assurance of imaging protocols.

1.5.1.8 Combining information from complementary imaging modalities or biomarkers

Few studies have integrated imaging-derived parameters from more than one imaging modality. In one LARC series, a pre-CRT dw-MRI ADC less than 1.06×10^{-3} had a sensitivity and specificity for predicting pCR of 100% and 88% respectively.⁴⁸ In the same cohort of patients, a reduction in $\text{SUV}_{\text{max}} \geq 40\%$ from baseline to after 10–12 fractions of CRT had a sensitivity and specificity of 100% and 75% respectively. When both these parameters' cut-offs were met, the sensitivity and specificity of pCR prediction increased to 100% and 94% respectively. When the baseline ADC threshold and a post-CRT reduction in $\text{SUV}_{\text{max}} \geq 76\%$ were combined, all pCR and non-pCR were correctly identified. In a separate study, using a relative change in a parameter made up from combination of pCT-defined blood flow and SUV_{max} led to an accurate prediction of a good treatment response with a ROC curve AUC of 0.955, suggesting it may be a useful combination of parameters to take forward for further study.⁸⁵ A correlation between intratumoural FDG-PET uptake heterogeneity and pCT measures of perfusion suggesting that heterogeneity in FDG signal intensity may offer information on tumour vascularity.⁸⁶

To improve risk stratification before initiating CRT for LARC, addition of a molecular biomarker to imaging parameters should be considered. Pre-CRT FDG-PET $\text{SUV}_{\text{max}} \geq 8.4$ in LARC had sensitivity and specificity both of 79% in one study. However, if this threshold was met and there was evidence of vascular endothelial growth factor receptor 2 on a pre-treatment tumour biopsy, sensitivity and specificity increased to 89% and 79% respectively.⁸⁷ Including carcinoembryonic antigen quantification further improved the predictive accuracy of post-CRT SUV_{max} in predicting pCR.⁵⁹

These findings support a multiparametric, multimodality approach to response-prediction modelling. It could also be inferred that models that do not rely on single point values may have a more robust predictive power.

1.5.2 Radiotherapy target selection

Accurate GTV definition is essential in radiotherapy planning to reduce geographical misses and limit the involvement of normal tissues in the treatment volume. Incorporating functional imaging into GTV delineation is attractive for a number of reasons – not least to reduce intra- and interobserver variability. It may also allow an increased automation of the target delineation process. Integration of information on intratumoural heterogeneity may allow for radiotherapy dose modulation across the GTV (as in Figure 4). A first step in the individualisation of CRT may be to use functional imaging to identify a BTV that may benefit from radiotherapy dose boosting.

Only one series has looked at the impact of functional imaging on GTV definition in pancreatic cancer. In a cohort of patients with LAPC, a CT-defined GTV was used as the reference volume for comparison with a GTV that was delineated following fusion of the FDG-PET with the planning CT. PET was also the most commonly used imaging modality for target volume definition in LARC. The use of parameter maps generated from other imaging modalities to delineate BTVs warrants further investigation. It is, perhaps, unsurprising that incorporating FDG-PET into radiotherapy planning improved interobserver agreement.^{54,88} A greater understanding of how to integrate information from functional imaging into radiotherapy planning is required. Some of the areas that require further investigation will now be discussed.

1.5.2.1 *Image segmentation*

Segmenting a digital image is a means of subdividing an image that may involve simplifying the information in the image into something that is easy to interpret or use practically. In the case of PET imaging, for example, it may be partitioning areas of the image into regions that demonstrate tracer uptake and those that do not. There are a number of ways of segmenting a PET image to generate a target volume. As there is no clear cut-off between a background level of uptake and pathologically significant uptake, a means of segmenting a volume that is thought to contain clinically significant tracer retention is required. A threshold-based method that is based on a specific signal-to-background ratio or as a proportion of SUV_{max} is commonly used. In the one published series in LAPC, FDG-PET was used to aid target volume definition. An $SUV \geq 42\%$ of SUV_{max} was used to auto-delineate the volume. The PET-derived GTV was larger than that defined using CT alone by 29.7%, due to extension of primary tumours and additional nodes (an example of how a previously unrecognised pathological lymph node was identified by FDG-PET can be seen in Figure 5. This lymph node should be included in the GTV). As there are no published data on the correlation of functional imaging with histopathology of LAPC, the best imaging threshold to define a GTV or BTV is not yet known. An example of how to use different uptake values to auto-segment the FDG-PET image to generate a GTV in LAPC can be seen in Figure 6. The potential limitations of FDG-PET in LAPC include the failure of FDG uptake to differentiate between tumour glucose metabolism and uptake in inflammatory cells. FDG-PET is therefore unlikely to be used in a fully automated method of delineating a target volume. This will be discussed further in Chapter 2.

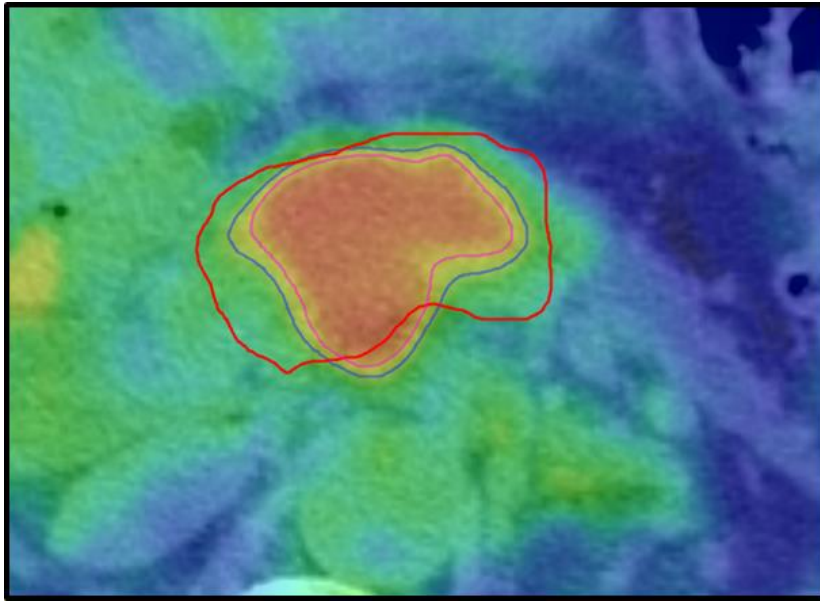


Figure 6: Magnified view of a trans-axial section through a locally advanced pancreatic adenocarcinoma. The GTV (red) was agreed by two radiation oncologists using only the planning contrast-enhanced CT. Following registration with the FDG-PET, it can be seen that FDG-avid tissue extends beyond the GTV. A semi-automated process can produce volumes outlined by 40% of the SUV_{max} (blue) or 50% of the SUV_{max} , which may facilitate accurate GTV definition.

Another approach to segment a PET image to generate a radiotherapy target volume is the gradient-based segmentation method (GBSM), which identifies the boundaries of a ROI by looking at gradient intensity crests within the image. It is thought that the GBSM is more accurate than a threshold-based approach.⁸⁹ These methods generate volumes of different sizes when used on FDG-PET images in LARC, with the GBSM generating smaller volumes.⁹⁰ If the PET-avid subregion of the tumour decreases during treatment, a decrease in PET-derived parameters such as SUV_{max} may be exaggerated as a result of PVE. This may have an impact on TVs derived from these parameters. It has been suggested that the GBSM may be less influenced by PVE than a threshold-based approach, increasing confidence in the target volume derived from PET, particularly if the uptake volumes are small.

1.5.2.2 Is the functional imaging modality providing radiobiologically significant information?

If functional imaging is to be used to generate BTVs for dose escalation, it should inform about a physiological characteristic that is associated with radioresistance. There may then be benefit in increasing the radiation dose to this subvolume. The most obvious region that may benefit from radiotherapy dose escalation would be an image-derived hypoxic volume. ¹⁸F-fluoroazomycinarabinofuranoside (FAZA) PET has been shown to be feasible in defining hypoxia in rectal cancers, although some correction for scatter from the bladder is required.⁹¹ Using FMISO-PET to derive a BTV for use as a target volume is not straightforward as there is high activity of the tracer within the normal rectal wall and in the bowel lumen.^{92,93} A greater understanding of FMISO tracer kinetics in rectal cancer is also required.

There has only been one report of the use of FMISO in pancreatic tumours.⁹⁴ In this study, only two out of seven pancreatic adenocarcinomas (stage not given) demonstrated FMISO uptake. This finding should be interpreted with caution as FDG-PET was used to define the ROI for FMISO uptake quantification. The threshold of FMISO uptake activity above that seen in normal muscle used to define hypoxia was not recorded. Also, the FMISO-PET imaging was performed 2 h post tracer injection, when it has been demonstrated preclinically and in head and neck studies, that waiting until 4 h post injection improves the signal-to-background ratio.^{95,96}

It has been suggested that FDG-PET may inform about tumour hypoxia as an increase in glucose transporter expression is seen in hypoxic cells.⁹⁷ Preclinical data has suggested that FDG-avid tumours require an increased radiation dose to improve rates of local control.⁹⁸

Areas of high FDG uptake on baseline imaging also seem to predict areas of residual metabolic activity post CRT in lung tumours.⁹⁹ Similarly in LARC, 70.6% of the voxels with an SUV > 50% of SUV_{max} were still metabolically active post CRT compared with only 51.1% of those with an SUV < 50% of SUV_{max} on baseline imaging.¹⁰⁰ This suggests that regions with a high baseline FDG-SUV may benefit from radiotherapy dose escalation. However, FMISO-PET-derived hypoxic volumes do not correlate with FDG-PET-derived volumes in LARC.⁹² The relationship between glucose uptake and regions of hypoxia requires further investigation.

1.5.2.3 Practicality and spatial evolution of a functional imaging-derived BTV

The ideal BTV for dose escalation would be located within the GTV and small enough for dose boosting without increasing the dose to surrounding normal tissues. FDG-PET-defined target volumes in LARC are typically smaller than MRI- or CT-defined GTVs.^{90,101,102} In one series, when the FDG-PET-derived and MRI-derived volumes were combined, the median increase in volume was 11% (range 0.5–72%), suggesting a good agreement between the volumes in the majority of cases. Another series that used a deformable registration algorithm noted an approximate mismatch of 50% between a FDG-PET-derived BTV and an MRI-defined target volume.⁹⁰

Due to the unpredictability of how a functional imaging-derived BTV will evolve during CRT, it is possible that the degree of agreement between the BTV and GTV will change. When the level of agreement between a volume derived from FDG-PET in LARC two weeks into CRT was compared with a baseline FDG-PET volume, a mean of 39% of the target volume defined using FDG-PET at 2 weeks was outside the baseline FDG-PET TV (range 6–100%).⁹⁰ This either suggests a variation in the spatial distribution of areas of FDG uptake, or a failure

of accurate image registration between time-points, perhaps because of changing rectal volumes during treatment, or peristalsis, which cannot be easily controlled. It also suggests that if functional imaging is to be used to inform the delivery of a non-uniform dose distribution, there will be a need for adaptive planning during the course of CRT to avoid the need for the addition of large margins to the BTV to ensure the volume receives the higher dose despite geographical variation.

1.5.2.4 Combining information from contemporaneous imaging studies

The best way to integrate complementary information from different functional imaging modalities to generate a BTV for dose escalation in CRT is not yet known. Parameter maps based on measures of hypoxia, proliferation, metabolism and perfusion could be combined to generate a volume that will contain any potential radioresistant cells. This approach requires validation of the imaging modality and an accurate means of image registration. An illustrative radiotherapy planning CT can be seen in Figure 7. The GTV and two BTVs derived from two different functional imaging modalities can be seen. It is not clear if the GTV should be extended to include the volume outside of the volume defined on registered CT and MRI conventional imaging. This has been proposed when FDG uptake is seen to extend beyond the anatomical imaging-defined TV.¹⁰²

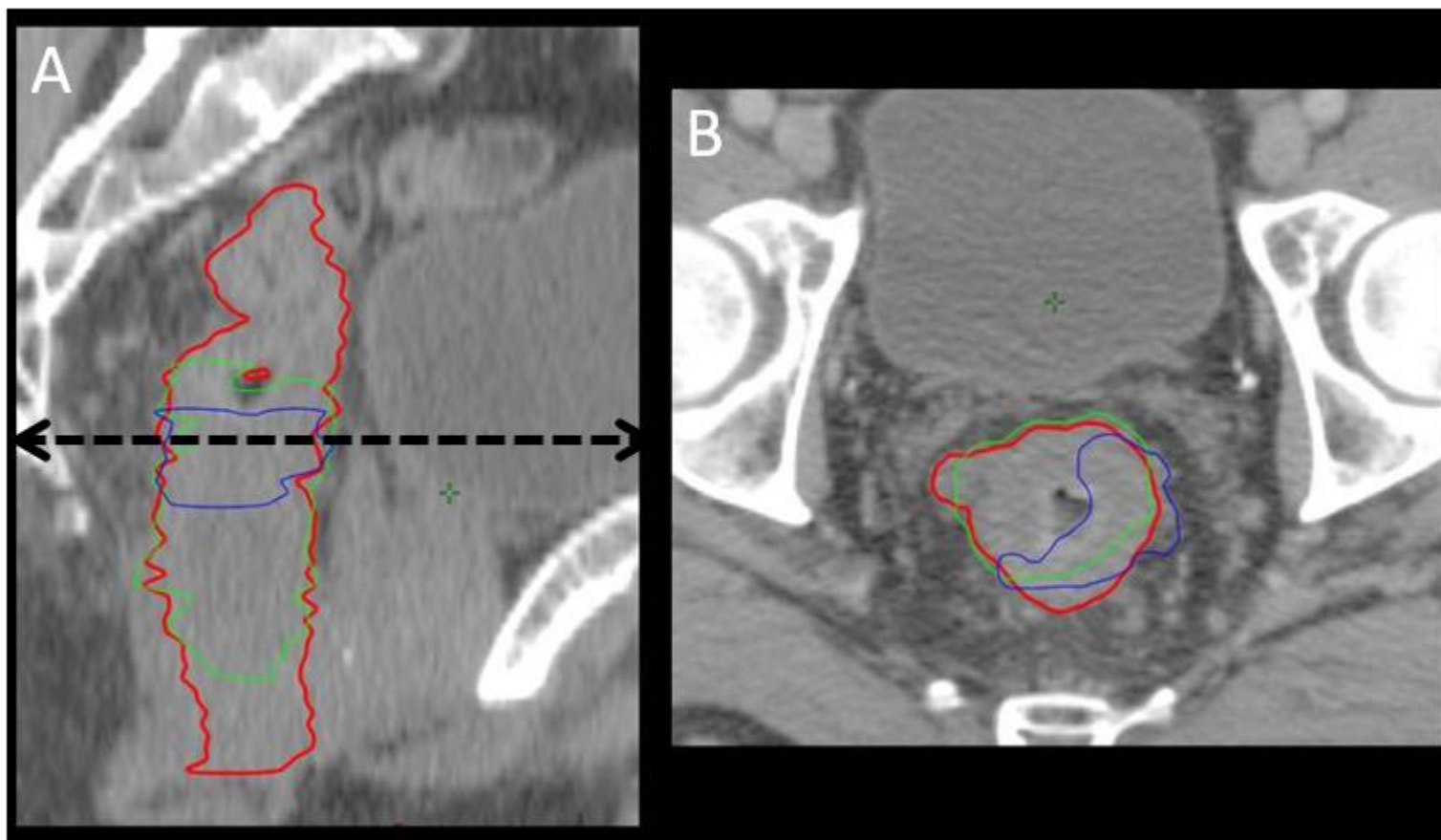


Figure 7: Sagittal (A) and axial (B) views through a rectal tumour as seen on a planning CT. The GTV (red) and two BTVs derived from different imaging modalities (BTV1, green, and BTV2, blue) can be seen.

One study looked at how target volumes from FDG, FLT and FMISO-PET in the same patient with LARC relate to one another.⁹² The mean volume of the target volumes derived from baseline FDG, FMISO and FLT-PET were 14.6 cm³, 5.2 cm³ and 9.2 cm³ respectively. How these volumes relate to the CT-defined GTV is not made clear. Because of the challenges associated with FMISO-PET acquired 3 h after tracer injection, FDG-PET was used to define a ROI, with extension of FMISO uptake extending beyond this volume only being included if the uptake was in a portion of the CT that looked like tumour.⁹⁰ Even though the areas of FMISO uptake extended beyond the FDG-derived ROI in every patient, the FMISO-BTV was significantly smaller than the FDG-BTV. There was no difference between the FDG- and FLT-BTVs. Target volume decrease during CRT was 54% for FDG-PET, 66% for FMISO-PET and 58% for FLT-PET. Using FDG-PET as reference volume, FLT and FMISO had a mean mismatch of 25% and 65% at baseline and 56% and 65% during CRT. The maximum distance of the furthest area of uptake from the point of intersection of FDG-PET and other volume was 6 mm for FLT and 14 mm for FMISO pre-CRT. These findings add further evidence that FDG uptake is not a reasonable surrogate for hypoxia or cell proliferation. Further work on how to integrate this complementary information into a deliverable radiotherapy dose prescription is needed.

Modelling studies may inform on how parameters obtained from functional imaging relate to radiosensitivity within an image voxel and can then be used to produce an individualised radiotherapy dose prescription. Combining information from more than one imaging modality may further improve these predictive models. This may give some indication as to whether, as can be seen in Figure 7, a boost volume should be based on the BTV derived from one functional imaging modality over another. It may be that the union of discrete

BTVs should be boosted, or the greatest benefit may be gained from boosting the volume of intersection between BTVs from different imaging modalities. This will vary depending on the imaging modalities employed. The best approach is not yet known, but the resultant volume needs to be deliverable without causing excess toxicity, an accurate means of image registration is required and a confidence in converting the poor spatial resolution of PET into an accurate target volume is still needed.

1.5.3 Literature review conclusions

To improve CRT treatment outcome in locally advanced pancreatic or rectal cancer, therapy needs to be individualised. Improving outcomes from biologically adapted CRT should result in increased rates of local control and, in LARC, increase in the incidence of pCRs and therefore reduced requirement for radical surgery, especially abdominoperineal resection. A robust means of stratifying patients with LAPC and LARC to be treated with CRT and its future biologically based modifications that can be applied across centres needs to be established. The use of non-invasive imaging to assess certain tumour phenotypes (e.g. hypoxia) is appealing as a replacement for an invasive biopsy. It also gives information about TV. The assessment of intratumoural heterogeneity can also be repeated. The best imaging modality or combination of imaging modalities and the optimum imaging time-point before or during CRT to perform this stratification assessment is not yet clear. The use of functional MRI is particularly attractive as there is no additional radiation dose incurred and there is wide availability in most hospitals. Re-assessing early in the course of CRT shows promise and may allow for adaptive, individualised therapy. Using a relative response index between time-points appears to be more accurate than absolute single time-point parameter quantification. Validation of any proposed predictive parameters on

separate cohorts is essential. Clear quality assurance of imaging protocols across hospital sites to allow for reproducible parameter calculation will be essential if imaging parameters are going to be used in population-wide risk stratification and target volume definition. A greater understanding of the radiobiological significance of functional imaging-derived parameter maps is required on a voxel-by-voxel level in order to produce an individualised radiotherapy dose prescription. Contemporaneous knowledge of the tumour's molecular characteristic biology will complement and aid the accurate interpretation of functional imaging. A multimodal assessment of physiological heterogeneity within tumours alongside evaluation of novel approaches to radiotherapy and biologically based radiosensitisation may ultimately lead to a targeted, personalised therapy that will improve treatment outcomes.

CRT for LAPC or neoadjuvant CRT for LARC is typically given over a period of 5–5.5 weeks, so an early response assessment would have to be within the first week or two to allow for any proposed treatment modification. The optimal time-point to perform this risk stratification to identify patients who may benefit from an increased radiotherapy dose is also not yet known. In HNSCC, persistent hypoxia measured by FMISO-PET at the end of the second week of CRT identifies patients at higher risk of local recurrence more accurately than pre-CRT imaging.¹⁰³ The optimal time-point to reassess treatment response in LAPC or LARC is not yet known.

Further studies are required to increase the confidence in the predictive and prognostic power of functional imaging in LAPC and LARC. A multimodality, multiparametric assessment of the tumours at more than one time-point to increase the likelihood of

finding predictive indices should be systematically explored. Attempts should be made to reduce the time from imaging to the start of CRT. The imaging modality used should give information about a physiological process that is associated with treatment resistance. With increased confidence in this imaging modality, the functional imaging could then be used for biological target volume definition. Delivering a higher radiotherapy dose to areas of the tumour that are less likely to respond, or integrating physiological modulating agents into the CRT regimen, may increase the likelihood of pCR without increasing treatment toxicity. This approach would, however, require a robust means of risk stratification that can be performed early in the treatment schedule.

1.6 Hypoxia as a cause of chemoradioresistance

Evidence that hypoxic conditions lead to radioresistance in cancer cells was first published in 1953 by Gray *et al.*⁸ This is due to the intrinsic dependence of radiotherapy on cellular O₂ to cause lethal damage to the tumour cell. Lack of oxygen in cells means that radiotherapy generates fewer reactive oxygen species within the cell and therefore fewer cytotoxic incidences of DNA damage. Hypoxia also leads to a more aggressive phenotype. The activation of gene expression as a result of hypoxia (via the hypoxia-inducible factor 1 α , HIF-1 α) leads to increased cancer cell survival and enhanced metastatic behaviour (reviewed by Lu and Kang¹³).

Poor vascularity characterises more than half of human tumours regardless of histology and grade.⁹ As well as variable distances from vessels to tumour cells, new blood vessels are structurally abnormal and precarious. Because of abnormal tumour vasculature and rapid cell turnover, the tumour microenvironment contains regions of normoxia, chronic hypoxia and acute hypoxia.

Chronic tumour hypoxia can also be termed 'diffusion-limited hypoxia' as a result of cells being too far from a viable blood vessel. The diffusion distance of oxygen in tumours varies according to cell type and oxygen consumption, but is in the order of 100–200 μm .¹⁰⁴⁻¹⁰⁶ Rather than there being clearly defined boundaries between areas of oxygenated and hypoxic areas of tumours, there is a continuum of oxygen tensions as the distance from the blood vessel increases. This has been demonstrated experimentally using the nitroimidazole EF5, where flow cytometric analysis of EF5 binding demonstrated a continuous range of cellular oxygen levels in subcutaneous xenograft tumour models.¹⁰⁷ Chronic hypoxia in tumours can also be the result of a long-term failure of oxygen delivery to the tumour. A clinically relevant example of this might be co-existent anaemia leading to a reduction in oxygen transport to the tissues.

Acute hypoxia may also be termed 'perfusion-limited hypoxia' as it arises from the intermittent opening and closing of tumour blood vessels.¹⁰⁸⁻¹¹¹ Two further subgroups of acute hypoxia have been proposed: ischaemic hypoxia and hypoxaemic hypoxia.¹¹² Ischaemic hypoxia results from the temporary shut-down of flow within the blood vessel. This can happen because of obstruction of the vessel lumen by blood constituents, fibrin, or tumour cells. It can also happen as a result of vascular remodelling. Acute hypoxaemic hypoxia results from a depletion of the oxygen content of the vessels as a consequence of sluggish or reversible blood flow within the tumour blood vessels or a reduction in red cell flux through the vessel.

Tumour hypoxia, and its associated radioresistance, leads to an increased likelihood of treatment failure. For example, in a series of patients with HNSCC treated with

radiotherapy, disease-free survival (DFS) rates of 77% were seen in patients with a median tumour oxygen tension of >10 mmHg compared with only 22% in patients in whom the median tumour oxygen tension was <10 mmHg.¹¹³ This is likely to reflect a failure of locoregional control as a result of tumour hypoxia. Two-year locoregional control was only 30% in those with HNSCC treated by radiotherapy with a pre-treatment tumour median oxygen tension of <10 mmHg compared with 73% in those with a median tumour oxygen tension >10 mmHg.¹¹⁴ Patients with HNSCC with more hypoxic tumours have poorer OS than those with less hypoxic tumours.¹¹ Further evidence that hypoxia leads to treatment resistance and poor survival comes from clinical trials in cervical and prostate cancers.^{115,116}

It is then perhaps surprising that previous large-scale Phase III trials of hypoxia-targeting therapies have failed to demonstrate an improvement in treatment outcomes. For example, while early phase clinical trials of the selective hypoxic cell cytotoxin tirapazamine in addition to CRT for HNSCC were favourable,¹¹⁷ the Phase III trial failed to demonstrate an improvement in overall survival or time to local recurrence.¹¹⁸ This could be because of a failure to select only those patients with a significant hypoxic tumour burden for treatment with hypoxic cell sensitisers.^{119,120} A means of detecting tumour hypoxia that is acceptable to patients would need to be utilised. A small substudy of the tirapazamine study showed that in a selected subgroup of patients with significant hypoxia, as shown by FMISO-PET, a clinical advantage was demonstrated with tirapazamine.¹²⁰ This same pattern of only seeing an improvement in treatment outcome with hypoxia-targeting strategies in those who demonstrate a significant hypoxic burden has been repeatedly demonstrated in HNSCC. For example, the hypoxic radiosensitiser nimorozole was only shown to be of benefit in patients with hypoxia as demonstrated by elevated circulating

osteopontin (OPN), a marker of hypoxia, or gene expression characteristics.^{119,121,122} There is a need for a means of characterising tumour hypoxia that is acceptable to patients to help guide treatment decisions.

1.7 Characterising hypoxia in human tumours

There are several techniques that can be used to measure tumour oxygenation. The available techniques can be described as being invasive or non-invasive. The methods either provide real oxygen measurements (e.g. polarographic methods) or measures of oxygen-sensitive markers (e.g. immunohistochemistry [IHC] of HIF-1 α on tumour biopsies). These markers of hypoxia can then be described as endogenous (usually hypoxia-inducible proteins or gene expression patterns) or exogenous (e.g. pimonidazole). A detailed review of all of the measures of tumour hypoxia is outside the scope of this thesis and has been reviewed elsewhere.¹²³ The following discussion will therefore focus on the most commonly used markers of tumour hypoxia leading into a discussion of clinical imaging of hypoxia.

1.7.1 Polarographic needle electrodes

The current gold standard is to introduce fine-bore oxygen polarographic electrodes (Eppendorf electrodes) into the tumour under local anaesthesia and to measure the partial pressure of oxygen directly. The required technique was first described in 1986.¹²⁴ Needle electrodes have been used to demonstrate intra- and intertumoural heterogeneity in oxygen levels in both preclinical models and human tumours.^{123,125} The probe is under computer control while multiple pO₂ measurements are taken. A stepping motor advances the probe automatically by small distances followed by a 0.3 mm retraction to minimise artefact from the effect of pressure on the sensing probe membrane. As the probe tracts

are blind, MRI, CT, or ultrasound visual guidance is required to differentiate viable tumour from necrotic foci within the TV. Hypoxia, as defined by needle electrode measurements, has been shown to correlate with poor radiotherapy treatment outcomes in a number of tumour types, including HNSCC and cervical cancer.^{11,126} Needle electrode measurements are, however, limited to superficial, easily accessible tumour sites, are invasive and can cause patient discomfort and bleeding, and require the additional use of patient imaging to identify if the electrode tip is in viable tumour tissue at the time of sampling. As readings are only taken along the needle track, this method does not provide three-dimensional (3D) information that could be used to define a radiotherapy target volume.

1.7.2 Endogenous markers of hypoxia

The detection of genes and proteins involved in the tumour cell response to hypoxia is another means of characterising the oxygenation status of tumours. These methods are limited by their invasive nature, their restriction to tumour sites amenable to biopsy or access to archival tumour material for later analysis, and correlation with patient outcomes. For example, the quantification of IHC of the endogenous markers of hypoxia carbonic anhydrase IX (CAIX) and HIF-1 α as well as hypoxia metagene analysis has been shown to be prognostic in a variety of tumour types.¹²⁷⁻¹²⁹

HIF-1 α is a key-regulator of cellular oxygen status. It is constitutively synthesised, but in normoxic conditions it is targeted for rapid proteosomal degradation.¹³⁰ In hypoxic cells, this process is suppressed, leading to upregulation of HIF-1 α .¹³¹ The HIF-1 α and HIF-1 β subunits can then heterodimerise to generate HIF-1. This molecule is known to activate a number of genes involved in hypoxic cell adaption and survival. The use of HIF-1 α for

characterising tumour hypoxia is limited by its short half-life and instantaneous response to hypoxia.¹³² These rapid changes in cellular HIF-1 α may explain the lack of correlation between HIF-1 α and pimonidazole in both preclinical models and in HNSCC.^{133,134}

CAIX is an enzyme concerned with the stabilisation of intracellular pH. While cell surface expression of CAIX is to be expected in some cells from the gastrointestinal tract,¹³⁵ it has also been demonstrated that CAIX expression is regulated by HIF-1 in cancer cell lines.¹³⁶ It has also been demonstrated that CAIX-expressing cervical cancer cells are resistant to ionising radiation.¹³⁷ CAIX IHC also correlates closely with needle electrode-defined hypoxia in cervical cancer.¹³⁸ While some agreement in IHC of CAIX and pimonidazole has been demonstrated, there are also areas of mismatch.¹³⁶ It is thought this is because the upregulation of CAIX in response to hypoxia can take several hours.¹²³ CAIX is, however, a promising endogenous marker of hypoxia in human tumours as its expression is associated with lower rates of local control and OS following CRT for HNSCC.¹³⁹

Hypoxic cancer cells rely on glycolysis to meet their energy requirements. Glycolytic pathways are therefore essential for the survival of hypoxic cells. This was first described by Warburg in the 1950s.¹⁴⁰ Glucose transporters (GLUT) are expressed on the cell surface and facilitate glucose influx into cells for anaerobic glycolysis. GLUT-1 IHC has been demonstrated a long distance from blood vessels and next to areas of necrosis, and its expression is a predictor of metastasis-free survival in locally advanced cervical cancer.⁹⁷ While GLUT-1 expression has been shown to correlate with pimonidazole IHC,¹⁴¹ it does not correlate with needle electrode measurements of tumour oxygenation,¹⁴² which has put its usefulness as a marker of hypoxia under question.

OPN is known to be upregulated under hypoxic conditions via Akt-mediated signalling to a ras-activated OPN promoter.¹⁴³ Serum OPN has been shown to be a predictor of outcome in head and neck cancer (including response to a hypoxic cell radiosensitiser).¹²¹ There is also a correlation between circulating levels of OPN with needle electrode measurements of hypoxia.¹⁴⁴

Gene expression profiling has identified certain 'signatures' that are associated with hypoxia.¹⁴⁵ Use of hypoxia metagene analysis has been validated in breast¹⁴⁶ and head and neck cancers,¹¹⁹ but has not yet been evaluated on pancreatic or rectal cancer samples. In head and neck cancer, it has been demonstrated that only genes that are upregulated as a result of hypoxia offer prognostic information, whereas genes that are downregulated are not.¹⁴⁷

1.7.3 Exogenous markers of hypoxia

The endogenous markers of hypoxia are chemicals or drugs that are administered to patients and taken up by cells. These chemicals are only reduced under hypoxic conditions and therefore retained in hypoxic, but viable tissues. Once they are retained in cells, they can then be detected by IHC, meaning that a biopsy or resection of the tumour is still required for their detection. The most commonly used agents in clinical use are nitroimidazole compounds such as pimonidazole or EF5. These compounds are retained in cells with an oxygen tension of <10 mmHg.^{148,149} Pimonidazole is considered to be a reliable method for detecting hypoxia¹⁵⁰ and has been shown to have a role as a prognostic biomarker in HNSCC.¹⁵¹

1.7.4 Limitations of the invasive methods of measuring tumour hypoxia

While these methods are thought to inform about inter- and intratumoural heterogeneity in cellular hypoxia, their use in personalising CRT regimens in pancreatic and rectal cancer is limited by the need for tissue sampling. Pancreatic tumours are difficult to biopsy and a diagnosis of pancreatic cancer is often made on scanty material or cytology. The reliable use of endogenous or exogenous markers of hypoxia is therefore unlikely to be clinically feasible. Both pancreatic and rectal tumours are likely to have heterogeneous microenvironments, particularly with respect to hypoxia. The validity of a small tissue biopsy in developing a hypoxia-based predictive or prognostic biomarker will therefore always be questionable. If radiotherapy dose painting, with dose escalation to a hypoxic subvolume, is to be employed, a non-invasive means of mapping the location of tumour hypoxia is needed.

1.8 Imaging hypoxia

A variety of imaging modalities have been employed with the aim of quantifying and spatially locating hypoxic regions within tumours in a non-invasive way. The breadth of the imaging modalities that have been used to either directly image hypoxia or to infer hypoxia based on the absence of perfusion has been reviewed elsewhere.¹⁵² PET is the most commonly used imaging modality used to characterise hypoxia using a variety of radiolabelled nitroimidazole compounds. The work presented in this thesis uses FMISO-PET to assess tumour hypoxia. FMISO is the most extensively studied, and most readily available, hypoxia PET tracer.

1.8.1 ¹⁸F-fluoromisonidazole positron emission tomography

The first experience of FMISO-PET imaging in humans was published in 1992 in a cohort of patients with various solid malignancies.¹⁵³ Since then, FMISO-PET has successfully demonstrated hypoxia in head and neck cancer,¹⁵³ non-small cell lung cancer (NSCLC),¹⁵⁴ gliomas,¹⁵⁵ soft-tissue sarcoma¹⁵⁶ and prostate cancer¹⁵³ among a small series of other tumours. A study to assess regional hypoxia in renal tumours using FMISO-PET was unsuccessful because FMISO and its metabolites are predominantly renally excreted.¹⁵⁷

FMISO's selectivity for hypoxic cells needs to be considered and a reproducible method of scanning to maximise the sensitivity and specificity of the test needs to be validated. The mode of action of FMISO uptake and how it is specific for hypoxic cells will be followed by a discussion of the methodological challenges of FMISO-PET scanning. The evidence of how the FMISO-PET-defined hypoxia can be used to predict response to treatment will be reviewed. As there has been interest in dose boosting partial TVs based on certain biological characteristics, the utility of FMISO in defining a BTV will be considered. The success of radiotherapy in tumour control is thought to be at least in part due to the effective reoxygenation of tumours during fractionated radiotherapy. The ability of FMISO to detect changes in tumour hypoxia over time will be discussed.

1.8.1.1 Mechanism of action of FMISO

FMISO is a nitroimidazole like pimonidazole. It is a lipophilic compound that enters the cell via diffusion. Once inside the cell, the nitro group is reduced by a number of enzymes, including cytochrome P450 reductase, xanthine oxidase and oxidoreductase. This process is summarised in Figure 8. If there is sufficient intracellular oxygen, the resulting radical

anion will be converted back to the intact nitrogroup and the compound will leave the cell via diffusion.¹⁵⁸ Under hypoxic conditions a sequence of reductions takes place, leading to ring fragmentation of the compound, covalent binding to macromolecules and retention of the tracer within the cell.¹⁵⁹ While some PET tracers have been demonstrated to dissociate from their radioactive label after injection (e.g. ¹⁸I-Iodoazomycin-arabinoside¹⁶⁰ or ⁶⁴Cu-ATSM), FMISO remains stable after injection with no metabolites of the compound being detected in the blood of mice or humans.^{161,162} As more than 80% of FMISO appears as the parent compound in the urine,¹⁶³ it is thought that there is no need to correct for metabolism of FMISO in scanning algorithms. FMISO has been demonstrated to be taken up by a variety of different tumours, whereas the uptake of ⁶⁴Cu-ATSM may be cell type-specific.¹⁶⁴

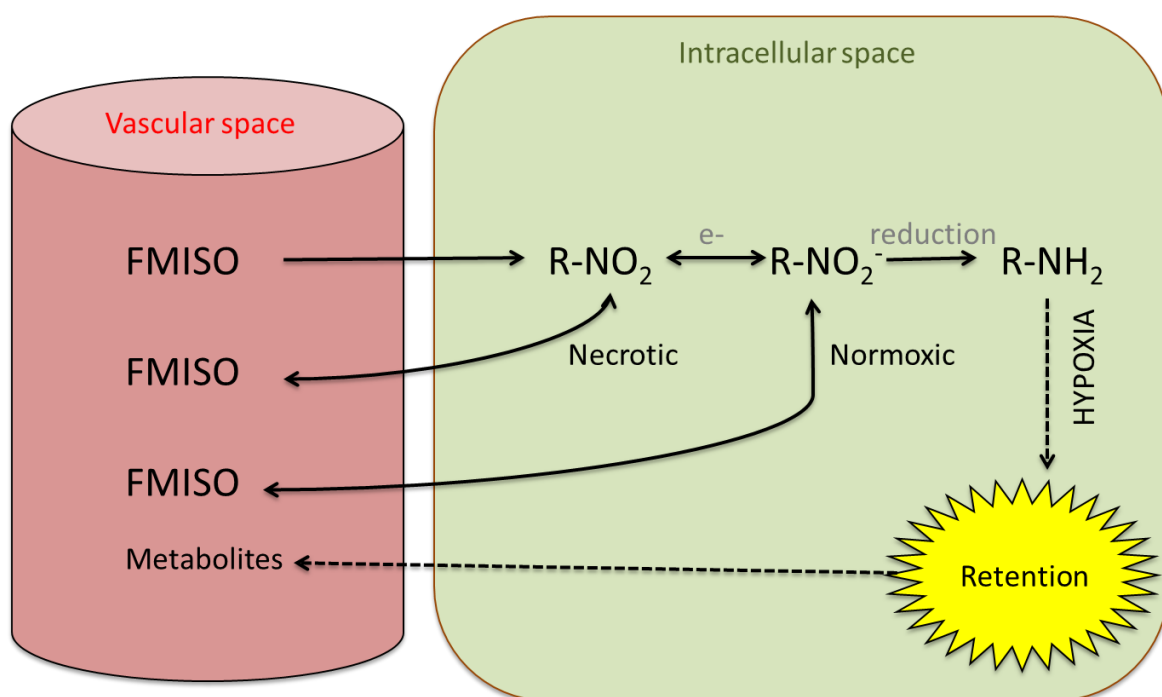


Figure 8: The mechanism of action of FMISO for the imaging of hypoxia. The FMISO molecule is sequentially reduced in the presence of hypoxia and retained in the cell. The molecule is free to diffuse back out of necrotic tissue and into oxygenated cells.

In an EMT6 spheroid model, misonidazole uptake was noted in areas next to necrosis.¹⁶⁵ Interestingly, misonidazole uptake was seen to follow an 'on-off' pattern, suggesting that the tracer is only retained in the cell once the intracellular oxygen level has fallen below a threshold level. Similar findings were seen using a monolayer cell sandwich system in which the only limitation of oxygen and nutrient provision to cells is the distance of diffusion.¹⁶⁶ Cells close to the oxygen supply demonstrated little ³H-misonidazole uptake. As distance from the oxygen and nutrition supply increased, so did ³H-misonidazole uptake. The standard deviation of the number of grains of ³H-misonidazole in each cell fell as the distance from the oxygen supply increased. This demonstrates a role for FMISO in defining chronic, diffusion-limited hypoxia.

The first *in vivo* validation of FMISO-PET using pimonidazole and CAIX staining as the reference standard used a rat rhabdomyosarcoma model.¹⁶⁷ Independent of tumour size, pimonidazole and CAIX staining was heterogeneously distributed throughout all tumour sections. FMISO SUVs > 1.4 that of normal tissues were able to demonstrate a hypoxic volume that correlated strongly with the pimonidazole- and CAIX-defined volumes. In a rat prostate tumour model, pimonidazole staining showed very close spatial correlation with FMISO autoradiography of tumour sections.¹⁶⁸

A dynamic assay of hypoxic cell survival raised a question of the sensitivity of FMISO uptake to define radiobiologically significant cells. The radiobiologically hypoxic fraction was derived from radiation response data in 36B10 rat gliomas.¹⁶⁹ *In vitro*, severely hypoxic cells were seen to steadily accumulate FMISO as a function of time up to 6 h, with the uptake

of FMISO being strongly dependent upon the availability of oxygen. The FMISO-derived hypoxic volumes in rats breathing air or 10% oxygen were smaller than the volumes anticipated from cell survival data. The authors suggest that this is because FMISO may only be taken up by the most hypoxic cells (those with a pO_2 of 2–3 mmHg). This opinion is supported by *in vitro* data that have shown that the oxygen level inhibiting FMISO binding by 50% relative to binding under anoxia varied from 0.55–1.75 mmHg in four cell lines.¹⁶² This implies that tumours with evidence of FMISO uptake have regions where the intracellular partial pressure of oxygen is less than 1–2 mmHg. Cells with a higher intracellular partial pressure of oxygen may not retain FMISO but could still in theory possess sufficient radioresistance to survive radiotherapy, putting them in a position to repopulate the tumour. This demonstrated that FMISO-derived hypoxic volumes could underestimate the volume of radiobiologically significant tumour hypoxia.

1.8.1.2 Validation of FMISO against needle electrode polarography and tissue perfusion

As the current gold standard of tissue hypoxia measurement is electrode pO_2 polarography, a number of validation studies comparing FMISO-PET with this method have been carried out. As electrode pO_2 quantification requires a superficial testing site, much of the work validating FMISO-PET in humans has been in HNSCC – particularly HNSCC nodal disease. As HIF-1 induces glucose transporter transcription, and hypoxia and enhanced glucose metabolism are negative predictors of survival, many of the studies have asked if FDG-PET could be used as a surrogate for hypoxia thus negating the need for another hypoxia-specific PET tracer. Studies in HNSCC have failed to show a correlation between FDG-PET and needle polarography.¹⁷⁰⁻¹⁷² FDG and FMISO uptake has been shown to be either mildly

correlated or not correlated at all in animal and human studies.¹⁷³ FDG-PET should therefore not be used as a surrogate hypoxia imaging study.

A high correlation has been observed between the ratio of FMISO uptake in a tumour ROI to the uptake in muscle (tumour:muscle, T:M) has been reported. In one study, the correlation was strong between FMISO T:M at the fraction of pO_2 polarography measurements ≤ 2.5 mmHg, ≤ 5 mmHg and ≤ 10 mmHg.¹⁷⁰ Other series have found that the most robust correlation exists between areas of high FMISO tumour-to-blood ratios (tumour:blood, T:B) rather than T:M.¹⁷¹ The highest correlation in this data set was between needle polarography readings and T:B when the data was acquired 2 h post injection of FMISO. This led to the blood FMISO activity taken at the time of PET scanner data acquisition as the reference for the activity seen in tumours to be widely accepted. A strong correlation was maintained even in the presence of two outliers – both of whom had predominantly necrotic malignant lymph nodes. It is therefore important to remember when comparing FMISO uptake as a measure of hypoxia with needle electrode measurements that FMISO is only taken up by viable cells. As areas of necrosis may be too small to be detected by the imaging modality chosen to guide the electrode, needle polarography can therefore overestimate the presence of radiobiologically significant hypoxia. This could explain findings seen in a C3H murine breast cancer model in which needle polarography defined hypoxic fraction correlated with TV but the FMISO-PET defined hypoxic volume did not.¹⁷⁴ It is also important to remember that needle electrodes offer a measure of extracellular oxygen tension that may bear little resemblance to what is happening intracellularly, particularly if there is tissue destruction and bleeding with multiple passes of the needle.

The relationship between hypoxia and perfusion is complex. Colour duplex sonography was used in addition to FDG-PET, FMISO-PET and needle polarography to assess a correlation between the markers of hypoxia and perfusion.¹⁷² While a correlation between FMISO T:B and pO₂ polarography was still seen, the sonographic measure of perfusion bore no correlation to needle electrode-confirmed hypoxia. This suggests that hypoxia is not caused by perfusion deficits alone but is multifactorial in origin. The correlation between hypoxic areas within tumours and perfusion is unclear. A study looking at nodal disease in HNSCC demonstrated a clear relationship between perfusion and hypoxia by showing a negative correlation between FMISO uptake and perfusion as ascertained by dce-MRI.¹⁷⁵ It should therefore not be assumed that a hypoxic volume can be defined simply by a lack of tissue perfusion.

1.8.1.3 Acquisition and interpretation of FMISO-PET images

There are a number of methodological challenges in acquiring and interpreting FMISO-PET images. A ROI for FMISO signal quantification needs to be delineated, which requires the accurate coregistration of images obtained from a complementary imaging study. The contrast of signal intensity to background activity that is taken to signify hypoxia is as yet undefined, making hypoxia volume definition challenging and threshold dependent. The washout of FMISO from normoxic cells and its retention in hypoxic cells is a dynamic process. The best time for data acquisition that optimises the contrast between hypoxia and background activity is still subject to investigation.

1.8.1.3.1 Defining a region of interest

As FMISO is able to passively diffuse throughout the body, some FMISO uptake can be seen in normal tissues. In early studies while scanning protocols were being developed, patients were immobilised for considerable amounts of time. As a result, uptake in normal muscle due to prolonged compression and transient ischaemia was observed.¹⁵³ This highlighted the need for another imaging modality to be used to define a ROI in which the FMISO activity could be quantified. Any background or spatially irrelevant FMISO uptake can then be ignored.

CT-defined regions of interest have been used successfully in HNSCC,¹⁷⁶ NSCLC and prostate cancer.¹⁷⁷ Many studies in HNSCC have relied on FDG-PET to delineate the TV.¹⁷⁸

A high background of FMISO activity is seen in rectal cancer. This is, at least in part, because of evidence of colonic excretion of the tracer.⁹³ Before determining if FMISO could be used to image hypoxia in rectal cancer the FMISO-PET images had to be coregistered with FDG-PET images. FMISO uptake within an FDG-derived TV was taken to represent significant hypoxia. Four out of six patients had detectable hypoxia within the primary tumour. One patient had evidence of FMISO uptake in a metastatic deposit with no hypoxia demonstrable within the rectal primary tumour. In another study that used FDG-PET to define a ROI for FMISO uptake in rectal cancer, all patients had FMISO uptake that extended outside of the ROI.⁹² CT images therefore also had to be used to see if this extension outside of the ROI remained in suspected tumour or not.

1.8.1.3.2 Image coregistration

In both animal models and clinical studies, the importance of accurate image coregistration cannot be underestimated, particularly if anatomical imaging is required to define a ROI. In animal studies, an atraumatic immobilisation technique that allows reproducible positioning has been developed.¹⁷⁹ In humans, particularly in HNSCC, FMISO imaging has been performed with the patient wearing immobilisation devices to replicate the radiotherapy treatment position and reduce variability in positioning.¹⁸⁰ Imaging software can then be used to coregister FDG-PET with FMISO-PET images.¹⁷⁸ A non-rigid modification of this registration based on CT densities limits inaccuracies of segmentation of the ROIs as a result of organ movement or differences in patient positioning.^{90,92}

1.8.1.3.3 Segmenting the FMISO-PET image

The need to define the level of FMISO signal intensity that is thought to be a significant representation of tumour hypoxia has been apparent since early clinical trials. As the diffusion of FMISO is ubiquitous, it is the contrast between the FMISO signal in hypoxic tissues and background that is important. There has been much debate as to how this background activity should be defined and measured and as to the level of FMISO uptake to background ratio that should be used to define uptake that represents tissue hypoxia.

When FMISO activity in the normal tissues of four animal species after FMISO scanning was pooled and quantified, the mean FMISO normal tissue to blood ratio was essentially 1.0 and 90% of the tissue to blood ratios were <1.3 .¹⁵³ This led early investigators to choose a $T:B \geq 1.4$ to represent significant hypoxia within the region of interest.¹⁵³ This was validated in human studies by demonstrating that the mean FMISO muscle to blood ratio was 0.98

with 87% of pixels in normal tissue having a $T:B \leq 1.4$.¹⁷⁷ In one animal model,¹⁶⁷ despite normal tissue having a mean SUV < 1.1 , a $T:B$ threshold value of ≥ 1.2 resulted in a hypoxic volume that was greater than calliper assessment of the whole TV. This was felt to be the result of the poor spatial resolution of the scan and led others to also recommend an SUV threshold of 1.4. A $T:B \geq 1.2$ has, however, been successfully used in soft tissue sarcoma.¹⁵⁶ The same threshold has been justified in the imaging of glioblastoma multiforme on the basis that brain tissue in the contralateral normal hemisphere had FMISO brain tissue to blood activity ratios of < 1.1 .¹⁵⁵ This suggests that the signal to background ratio that represents true tumour hypoxia may be cell type specific, with individual thresholds needed for different tumour types.

While blood is often used as the reference value to calibrate FMISO signal intensity, internal, image-derived values have been successfully used, including normal muscle and mediastinum.¹⁵⁴ This is advantageous as it minimises error in the timing and processing of blood samples. It also reduces the radiation exposure to staff as repeated blood sampling is not required.

One group investigating biological image-guided radiotherapy in rectal cancer advises against using a SUV approach altogether.^{90,92} In a subpopulation of their study group, using a SUV threshold value would have resulted in FMISO TVs that were larger than FDG-PET-derived volumes. This would have resulted in a hypoxic fraction of greater than 100% because FDG-PET was used to define the tumour ROI. They therefore recommend a gradient-based segmentation method. Gradient-based segmentation involves identifying the region of most rapid change (the crest) in signal intensity from background levels to

areas of high uptake. In this study, a large amount of image pre-processing was required, including clustering of tumour voxels. A background level was selected from an uninvolved site at the edge of the pelvis. The crest was automatically computed, and using this method no TVs had a hypoxic fraction of 100% or more than the TV. The impact of image pre-processing and reduction of noise within the image on the ability of the image to then accurately represent tumour hypoxia is not clear.

A clearer understanding of how FMISO uptake values derived from PET images relate to more direct quantification methods of hypoxia is needed.

1.8.1.3.4 The timing of image acquisition and dynamic FMISO-PET scanning

The best time-point to acquire the FMISO-PET image after tracer injection has varied between studies. Dynamic PET scanning involves the acquisition of serial PET images over time, deriving time activity curves (TAC) of tracer signal against time and performing analysis of the TACs to inform about tracer metabolism and therefore characterise the physiological process that is being imaged.

FMISO washout from non-hypoxic cells is slow. This can lead to a low contrast between hypoxic regions and normoxic tissues. However, delayed scanning could further reduce signal intensity because of the relatively short half-life of FMISO.

In a murine breast cancer model, PET emission data acquisition was continued for 5 h post injection of the tracer. The FMISO activity tumour to background ratio continued to increase up to 2 h post injection. After 2 h a steady state was reached which did not change for the remainder of the scanning period.¹⁷⁴ These findings were mirrored in the first series of FMISO-PET scanning in human malignancy to be reported.¹⁵³ Plasma FMISO

concentration peaked immediately after injection before being rapidly redistributed. This was followed by a slow clearance phase giving an FMISO half-life of 380 ± 50 min. Activity in normal muscle equilibrates with plasma by approximately 30 min, whereas selective retention is seen with tumours by around 1 h and this will then persist. This led the authors of this early report to recommend that data acquisition for FMISO-PET scanning is best left until 2 h or more post injection as this is when the T:B will be most marked.

A study in HNSCC obtained images found a higher FMISO-to-background contrast when images were obtained 4 h after injection rather than 2 h.⁹⁵ This is consistent with the findings of a study looking at spontaneous rhabdomyosarcomas in dogs.⁹⁶ This advocated the extension of scanning times to 4 h, as this was when the most useful information about hypoxia within the tumour was identified.

By scanning too early, it could be that the FMISO signal is simply a marker of perfusion rather than hypoxia. For example, when imaging the benign brain tumour meningioma, a rapid uptake of FMISO is seen immediately following tracer injection but no evidence of retention of the tracer is seen at later scans.¹⁶³ In a study of patients with HNSCC and NSCLC, the early phase of data acquisition was found to be very informative.¹⁵⁴ The FMISO TAC was plotted for the first 14 min following injection of the tracer. The ROI was then classified as being 'rapid washout', 'intermediate', or 'accumulation'. The FMISO activity in the 'rapid washout' category is likely to be due to perfusion of the tracer and little else. No patients in this category went on to experience tumour recurrence during follow-up.

Patients with HNSCC were studied in an attempt to develop a kinetic model of FMISO-PET scanning to understand the spatial and temporal distribution of hypoxia in tumour

tissue.¹⁷⁸ PET data acquisition started at the time of injection and a dynamic image acquisition was performed for 15 min in 9 patients and for 1 h in 7 patients. All patients then had static scans at 2 and 4 h post tracer injection. A $T:B \geq 1.4$ was used to signify hypoxia. A 12 mm margin was added to the FDG-PET TV to define a ROI. From this, the FMISO TAC could be plotted. This kinetic model assumes a two-compartmental model, with FMISO either irreversibly binding and being retained intracellularly or transiently diffusing through the ROI. Three different perfusion-hypoxia patterns were observed within the tumour ROI. Some areas show high perfusion, no hypoxia, others show perfusion and hypoxia, and some areas have reduced perfusion and hypoxia. FMISO kinetic parameter maps have not yet been used to delineate hypoxic regions within tumours for BTV definition.

1.8.1.4 Use of FMISO-PET to predict treatment response

While tumour hypoxia has been known to impact upon treatment outcomes, including OS, for some time, the use of FMISO scanning to detect hypoxia is relatively recent. Despite this, there are already data suggesting that FMISO-detected hypoxia can be used to predict treatment outcome. In one study of patients with HNSCC, FMISO SUV_{max} , hypoxic volume and nodal stage were all associated with patient survival, with a suggestion that SUV_{max} may be the strongest predictor.¹⁷⁶ Two patients in the first study to demonstrate a link between FMISO-PET findings and treatment outcome in HNSCC showed an increase in FMISO SUV_{max} when they were reimaged during treatment. This was thought to be sign of particularly poor prognosis as both went on to develop local recurrence within the radiotherapy treatment field and distant metastases during follow-up.¹⁸¹

In a study of patients with HNSCC and NSCLC, dynamic FMISO scanning has been used to predict response to treatment.¹⁵⁴ No patients with no FMISO retention 14 min after injection went on to develop recurrences, whereas 5 out of 6 of those who were accumulating FMISO at the end of this period did.¹⁵⁴ An SUV threshold that might be used to predict response to treatment was subsequently sought. A T:M ≥ 2 in HNSCC and a tumour-to-mediastinum ratio ≥ 1.6 in NSCLC on the PET image acquired 4 h post tracer injection predicted 10 out of 13 recurrences and also allowed all of the treatment responders to be identified. This was improved further by factoring in the SUV_{max} value observed within the ROI into the analysis. By doing this, 11 out of 13 treatment failures were predicted without diminishing the sensitivity of the test to identify treatment responders. When analysed as a single variable, SUV_{max} was a less robust predictor of treatment outcome. This observation was repeated in another study of FMISO-PET in HNSCC.¹⁷⁸ It has been suggested that the tumour-to-reference tissue uptake ratio may be more valuable in predicting response than SUV_{max} with FMISO-PET because of interindividual variation in FMISO clearance from non-hypoxic tissues. This hypothesis has not yet been tested.

Dynamic FMISO scanning can be used to predict response to treatment. In a study of HNSCC patients,¹⁷⁸ a 'malignancy value' was derived from a measure of tumour perfusion and the ratio of perfusion to tracer retention derived from FMISO TACs. This derived value correlated closely with DFS. The hypothesis put forward is that demonstration of high tumour perfusion prior to radiotherapy may equate to more efficient reoxygenation during fractionated radiotherapy. Hypoxic areas that have adequate perfusion could perhaps be thought of as having an 'intermediate risk' of local recurrence. The hypothesis that tumours

that have the ability to reoxygenate during treatment are most likely to respond to treatment is an interesting one.

The addition of the hypoxic cell cytotoxin tirapazamine (TPZ) to CRT in HNSCC initially showed very promising results in a Phase I trial.¹¹⁷ As no treatment stratification was performed prior to giving TPZ in the subsequent Phase III trial, a survival advantage could not be demonstrated with the addition of TPZ to CRT.¹¹⁸ A later subanalysis of the Phase II trial revealed that stratification based on FMISO-PET-defined hypoxia at baseline could predict response to CRT and CRT plus TPZ, with the greatest benefit being seen in patients with hypoxic tumours treated with TPZ.¹²⁰ This identifies a potential role for FMISO-PET in treatment strategy selection.

FMISO-PET may have a prognostic role in the management of high-grade gliomas. Both a greater volume of FMISO-PET-delineated hypoxia and maximum FMISO signal intensity were strongly associated with time to progression and overall survival in glioblastoma multiforme.¹⁸² While the contrast-enhanced MRI TV also correlated with treatment outcome, multivariate modelling confirmed the importance of hypoxic volume and SUV_{max} in predicting response to radiotherapy. Use of the multivariate Cox model showed that an increase in hypoxic volume of 7–8 cm³ was associated with a 50% decrease in survival, as was an increase in SUV_{max} of 0.25.

One series in HNSCC has observed that it is the failure of tumour reoxygenation during CRT that informs the most about patient outcome.¹⁰³ The prognostic power of FMISO-PET-derived parameters was seen to increase at 1 and 2 weeks into CRT compared with a

baseline assessment of hypoxia. This observation requires validation in a larger cohort of patients and in a variety of tumour types.

These studies suggest that FMISO-PET could be used to identify both those who would benefit from treatment intensification and a subgroup of patients who might derive benefit from hypoxia-targeting therapies.

1.8.1.5 Use of FMISO-PET to generate biological target volumes for radiotherapy planning

Applying high-precision radiotherapy techniques to dose escalate to a high-risk BTV requires accuracy in target definition, delineation and dose delivery. A reproducible and accurate method of outlining these hypoxic subvolumes therefore needs to be developed.

In animal models, autoradiography of tumours following FMISO-PET scanning has shown marked heterogeneity of FMISO uptake within tumours, with some central sparing in larger tumours, suggesting necrosis. Carbogen breathing markedly reduces FMISO uptake supporting the role of FMISO as a reporter of hypoxia.¹⁷⁴ While FDG uptake was shown to be homogenous throughout the tumour in a preclinical model using xenografts of a rat hepatoma cell line, FMISO uptake was heterogeneous and located adjacent to areas of necrosis.¹⁷³ It could therefore be the case then that FMISO uptake is scattered throughout the GTV, rather than in discrete subregions of the tumour, which would therefore require radiotherapy dose escalation strategies to boost the entire TV.

High-resolution imaging is required to give confidence in the use of FMISO-PET to define a BTV. In early studies, the spatial resolution of FMISO-PET was poor. Because a single surviving hypoxic cell could theoretically repopulate a tumour post CRT, investigators suggested that the application of FMISO-PET should be limited to defining a tumour as

‘hypoxic’ or ‘not-hypoxic’.¹⁵³ With improving technology, a pixel-by-pixel analysis of FMISO uptake became possible.¹⁷⁷ This is particularly important in tumours with small amounts of hypoxia. Hypoxic volumes within tumours have been reported to be as low as 0.2% of the TV. This is likely to have been missed if the entire tumour had not been analysed and the spatial resolution of the scanner was lower.

HNSCC is associated with high rates of local recurrence – often within the previously treated GTV. Radiotherapy dose-escalation to a high-risk BTV based on biological characteristics such as hypoxia would seem to be advantageous in reducing local recurrences. In HNSCC, no correlation between FDG uptake and FMISO uptake was observed with only 0.3–95.7% of the FDG-defined volume also showing areas of FMISO uptake on coregistered imaging.¹⁸³ The importance of waiting until 4 h post tracer injection before delineating the region of FMISO retention is supported by the observation that the spatial distribution of FMISO-defined hypoxic volume using an SUV threshold varies considerably between 2 h and 4 h post injection.¹⁵⁴

A feasibility study in HNSCC patients has demonstrated that the FMISO-PET-derived hypoxic volume could be dose escalated to 108% of the usual dose in 6 out of 8 patients using IMRT without compromising normal tissue constraints.⁹⁵ To do this, a software package was used to delineate any areas within the ROI with a FMISO tumour-to-cerebellum ratio of ≥ 1.3 . The two hypoxic volumes that could not be dose escalated were because of location of the tumour, not necessarily the size of the hypoxic volume. In another HNSCC radiotherapy planning study, the feasibility of dose escalating to the hypoxic volume seemed more promising.¹⁶⁸ A dose of 120% was deliverable to the hypoxic

subvolume without compromising normal tissue tolerance in all of the 10 patients. An attempt to boost the dose to 150% was successful in one patient. It is therefore suggested that in patients with a large TV with a small hypoxic fraction, dose boosting the hypoxic area to 150% of the total dose may still be possible. This line of thinking does, however, assume that the FMISO hypoxic is a static volume that can be reproducibly targeted throughout fractionated radiotherapy.

The variability in FMISO distribution in HNSCC was further investigated by repeating the FMISO-PET scan at baseline and again after 3 days.¹⁸⁰ This time interval was selected as it was thought to represent the typical time delay between radiotherapy planning scans and the initiation of treatment. When the TVs were matched and a voxel-by-voxel comparison of FMISO retention values was compared there was a strong correlation between scans in only 6 of the 13 patients. This would not support the use of FMISO-PET in delineating hypoxic volumes for radiotherapy dose escalation and highlights the need for a greater understanding of variation of FMISO retention patterns within tumours over time.

In NSCLC, reimaging with FMISO-PET after 1–2 fractions of radiotherapy did not detect changes in FMISO-PET-defined hypoxic volumes.¹⁸⁴ By the mid-point of radiotherapy, however, a difference could be detected as the median fractional hypoxic volume had fallen from 58% to 29% of the TV. While most tumours did demonstrate evidence of reoxygenation throughout treatment, others did not. It does not appear possible to predict *a priori* which tumours are likely to reoxygenate during radiotherapy and which are not. The impact of failure of the reoxygenation on treatment outcome requires further clarification.

There can be considerable changes in TV throughout fractionated radiotherapy as defined on anatomical imaging. In one study in HNSCC, patients with locally advanced oropharyngeal, oral cavity, laryngeal, or hypopharyngeal squamous cell cancers had the primary tumour imaged with MRI and FMISO-PET. The imaging was performed at baseline and after 4 weeks of treatment and the MRI-defined TVs were shown to shrink to approximately half their original size.¹⁸¹ The FMISO-PET-derived hypoxic volume decreased more markedly from a mean of 4.1 mL to 0.3 mL at 4 weeks. As the hypoxic fraction of the TV is decreasing more than the absolute TV, this would suggest a degree of reoxygenation. In a Phase I study, the addition of TPZ to CRT for HNSCC meant that by week 4 of treatment, no patients demonstrated any FMISO uptake. In the Phase II study, of the 29 patients who had evidence of tumour hypoxia at baseline, only 6 had evidence of residual hypoxia at 4 weeks.¹²⁰ Interestingly, in the group treated with TPZ in addition to CRT, only two had residual hypoxia when FMISO-PET was repeated 4 weeks into treatment. While the primary disease was adequately controlled in these patients, both went on to develop distant disease. This suggests that failure of hypoxia to resolve during CRT with the addition of TPZ selects out biologically poor prognosis disease that may benefit from intensified systemic therapies. This further emphasises the need for an adaptive approach to radiotherapy treatment planning and treatment strategy selection.

In glioblastoma multiforme, TV, as delineated on T1-weighted gadolinium-enhanced MRI, correlates closely in terms of overall volume and spatial distribution with areas of FMISO uptake.¹⁵⁵ FMISO-PET was also correlated with MET-PET. MET-PET is a marker of tumour aggressiveness as it is closely correlated with tumour grade and proliferative rate. While the FMISO-PET and MET-PET uptake correlated volumetrically, no spatial relationship was

observed. This reflects findings in a preclinical animal model which demonstrated spatial heterogeneity between FMISO-defined hypoxic regions and MET-PET scanning.¹⁷³ This highlights that the two PET tracers characterise different biological processes. Which of these processes would be most advantageously targeted in improving clinical outcome remains uncertain, but targeting both volumes in many cases is likely result in a BTV that is not much smaller than the GTV, requiring dose escalation to the entire TV, which may not be deliverable without causing significant normal tissue toxicity.

The use of FMISO-PET in LARC has been discussed previously. A high background activity within the rectum as a whole has been reported, which may prove a challenge if FMISO-PET were to be used to delineate a hypoxic BTV for radiotherapy dose escalation.^{92,93} An ability to quantify FMISO uptake in rectal cancer and to use it to define a BTV as well as to characterise changes in the hypoxic volume during CRT has been demonstrated in a series of 10 patients.⁹²

1.8.2 Implications for future studies that utilise FMISO-PET

There is convincing evidence from preclinical studies that cellular FMISO retention informs about cellular hypoxia. Histological validation of FMISO-defined hypoxia using known markers of hypoxia in human tumours has not been reported and is required to further increase confidence in the imaging technique prior to its integration into radiotherapy planning. An understanding of the baseline variability of FMISO-PET-derived parameter quantification is currently lacking. A robust means of delineating a hypoxic volume based on data from FMISO-PET is needed. For this hypoxic volume to be used to guide radiotherapy dose boosting, a confidence in the spatial stability of the hypoxic volume

within the tumour ROI during CRT would be required. The majority of the studies of FMISO-PET have been performed in HNSCC, presumably because of the ability to immobilise the patient to allow for accurate image registration and the ease of access to tumours and regional lymph nodes for needle electrode measurements of hypoxia. Before FMISO-PET should be used to guide treatment strategy selection in other tumour sites, an understanding of its predictive utility in each cancer type will be required. The observation by Zips *et al.*¹⁰³ that it is the failure of tumour reoxygenation at the end of the second week of CRT, as ascertained by FMISO-PET, that informs about patient outcome in HNSCC needs to be repeated and validated in other tumour types.

1.8.3 The ideal hypoxia imaging study

For an imaging modality to be used as a hypoxia-specific means of deriving a predictive or prognostic biomarker to guide treatment strategy selection or as a method for delineating a hypoxic BTV for radiotherapy dose escalation, a number of assumptions are taken and need to be proven before its clinical application. It should be possible to differentiate between areas of hypoxia and necrosis and regions of normoxia. It should be able to detect small changes in hypoxia and therefore identify areas of acute, perfusion-dependent hypoxia from areas of chronic, diffusion-limited hypoxia. It should represent intracellular oxygenation and not that of the interstitial fluid or intravascular oxygenation. It should be simple to perform and have low test-retest variability. Perhaps most importantly, it should identify all radiobiologically significant hypoxia.

1.8.4 Reducing tumour hypoxia by targeting intracellular signalling

An alternative approach to improving CRT outcomes following radiotherapy dose escalation might be to reduce tumour hypoxia. Inhibition of the phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) pathway has been shown to reduce tumour hypoxia in murine models. HIV protease inhibitors have been shown to have an anti-tumour effect which is thought to be mediated by the inhibition of phosphorylated-Akt (pAKT).^{185,186} Inhibition of pAKT is thought to reduce hypoxia in part due to a reduction in mitochondrial oxygen consumption.¹⁸⁷ A number of studies have also shown a reduction in tumour hypoxia and a demonstrable improvement in perfusion as a result of microvessel normalisation.¹⁸⁸⁻¹⁹⁰ Upstream signals to pAKT are known to be altered in pancreatic cancer and have been demonstrated to lead to radioresistance in preclinical studies.¹⁹¹⁻¹⁹³ While inhibition of pAKT, including with nelfinavir, has been shown to reduce hypoxia and stabilise perfusion in preclinical tumours,¹⁸⁸ this has not yet been demonstrated in the clinical setting.

1.9 Thesis outline

The work presented in this thesis explores the use of functional imaging to personalise CRT for LAPC and LARC. Central hypotheses to the work presented here include:

- Image-derived parameters; whether they are derived from functional or anatomical imaging modalities can be used to describe biological heterogeneity in LAPC and LARC and can therefore be used in CRT response prediction and for prognostication.

- Quantifying the response in a functional imaging-derived parameter may be a more robust metric on which to base response prediction algorithms than single time-point assessments.
- FMISO-PET can be used to accurately characterise tumour hypoxia, including quantifying reoxygenation of tumours during fractionated CRT, and to define a hypoxic BTV.
- There is heterogeneity in the rates of tumour reoxygenation during CRT and the failure of reoxygenation, as described by persistent FMISO retention, can be used to predict response to CRT.
- Functional imaging, including FMISO-PET, can be used to define a BTV for selective radiotherapy dose boosting.

The work presented in this thesis contributes to the aim of using functional imaging to personalise CRT in LAPC and LARC and ultimately improve treatment outcomes. As tumour hypoxia causes radioresistance, a method that targets hypoxia may impact positively on treatment outcomes. It could be that, as in HNSCC,¹⁰³ pancreatic and rectal tumours that fail to reoxygenate are at the highest risk of treatment failure.

The prognostic utility of FDG-PET in LAPC treated with definitive CRT was tested and the results are presented in Chapter 2. In a step towards applying FDG-PET to treatment strategy selection, the ability of FDG-PET to identify patients who may benefit most from local treatment intensification was explored. As the SUV_{max} is subject to noise, its relationship to other FDG-PET-derived parameters was investigated.

Chapter 3 concerns the application of functional imaging to derive BTVs for selective radiotherapy dose escalation in LAPC and to quantify changes in hypoxia and perfusion. The ability of pre-CRT FDG-PET to identify areas of residual metabolically active tumour post CRT was investigated. The test-retest variation in FMISO-PET-derived parameters was explored, as well as the spatial stability of FMISO uptake. Dynamic FMISO-PET and perfusion CT were then used to describe changes in hypoxia and perfusion as a result of nelfinavir, a putative hypoxia modifier, therapy.

Functional imaging tests are resource intensive and require additional hospital visits for patients. The use of anatomical imaging that is required for staging purposes and radiotherapy planning to risk-stratify patients and guide treatment strategy selection would reduce the imaging burden for patients at low additional cost. Texture features of grey-scale images that are thought to describe intratumoural heterogeneity have been correlated with patient outcomes in HNSCC and lung cancer.¹⁹⁴⁻¹⁹⁶ Chapter 4 describes the development of a software tool to extract texture features from clinical images and work to try to develop a signature of texture-based features that predicts response to CRT in LARC. A secondary question of whether pre-CRT FDG-PET SUV_{max} improves the predictive function of the texture-based approach was also tested.

Chapter 5 describes the development and preliminary results of a clinical study that aims to characterise hypoxia, perfusion and reoxygenation in rectal cancer and to correlate these with clinical outcomes. The RHYTHM-I (modulation of Radiotherapy according to Hypoxia: exploiting changes in the Tumour Microenvironment to improve outcome in rectal cancer) study aims to validate imaging techniques that describe hypoxia and

perfusion against histological markers. The ability of FMISO-PET, dce-MRI and pCT to quantify changes in hypoxia and perfusion during CRT was tested on preliminary data. A method of using circulating biomarkers of hypoxia and tumour biopsies, either immunohistochemistry of biopsy material or gene expression profiling, to quantify changes in hypoxia and perfusion was developed. Final results will be presented when the study has fully recruited. Ultimately, it is hoped that this complementary information can be combined to produce a robust response prediction algorithm that can be used to direct treatment strategy modifications.

This thesis concludes in Chapter 6 with a brief summary of results and a discussion of some of the areas for future study that have arisen from this work.

Chapter 2: The application of pre- and post-chemoradiotherapy FDG-PET/CT to treatment strategy selection in locally advanced pancreatic cancer

2.1 Introduction

This thesis investigates the application of functional imaging to the personalisation of CRT strategies. Patients with LAPC often fail in distant sites following locally intensive therapy. Robust identification of those patients who would most benefit from local treatment intensification would help guide treatment strategy selection. The aims of the work in this chapter are:

- To investigate the relationship between FDG-PET SUV_{max} and SUV_{peak} , SUV_{mean} , SUV_{median} and TLG in LAPC.

Because SUV_{max} is subject to considerable noise, it was postulated that there would only be a weak correlation between SUV_{max} and other FDG-PET-derived parameters. The ability of each of the SUV parameters to predict patient outcome was investigated. The null hypothesis to be tested states that there is no correlation between SUV_{max} and these parameters.

- To understand the correlation between SUV_{peak} , SUV_{mean} and SUV_{median} and TLG in LAPC.

If each of these parameters is closely correlated, it may be that future studies that aim to assess the predictive and prognostic utility of FDG-PET in CRT-treated LAPC do not need to calculate all of these parameters. The null hypothesis to be tested states that there is no correlation between these parameters.

- To see if FDG-PET parameters and CA19-9 can identify patients who will only ever have cancer localised to the pancreas and, conversely, those who metastasise early following CRT.

SUV_{max}, SUV_{peak}, SUV_{mean}, SUV_{median}, TLG and CA19-9 values pre- and post CRT and their relative change from pre- to post CRT were compared between those who only ever have local disease and those experience metastatic disease and those who metastasise early (within 6 months of study entry). This was to investigate if any of these variables are useful in predicting the pattern of disease progression on an individual patient basis. CA19-9 was included as it was thought that a multimodality algorithm to predict the pattern of disease progression may be more accurate than each independent variable used in isolation. The null hypothesis to be tested states that there is no relationship between FDG-PET-derived parameters or CA19-9 and patterns of disease progression following CRT.

- Previously identified, patient-specific FDG-PET parameters (pre-CRT SUV_{max} < 3.5 or a $\% \Delta \text{SUV}_{\text{max}} > 60\%$ from pre- to post CRT)⁴⁴ were applied to this cohort of patients to assess their universal prognostic utility. Variable thresholds identified from this specific patient population were also tested to see if they more accurately describe OS.

A secondary hypothesis to be tested was to see if a relative change in SUV_{max} will be more informative about OS than single time-point (pre- and post-CRT) SUV_{max}. The null hypothesis would state that there is no relationship between FDG-PET-derived parameters and OS.

2.2 Methods

2.2.1 Patients

Patients recruited to the ARC2 clinical trial, a Phase II study looking at the addition of nelfinavir to CRT for LAPC, were included in this analysis. All patients had histologically confirmed locally advanced pancreatic adenocarcinoma. Only patients who completed treatment as outlined previously¹⁸⁶ and in Chapter 3 were included in this per-protocol analysis.

2.2.2 FDG-PET/CT acquisition

FDG-PET/CT was performed at baseline and 6 weeks after completing CRT. The intended imaging schedule is outlined in Figure 9. All scans were performed on a GE Discovery 690 (GE Healthcare, Buckinghamshire, UK). After fasting for 6 hours and ensuring that the blood glucose was <10 mmol/L, FDG was injected at a dose of 4 MBq/kg (up to 600 MBq). PET acquisition was started after an uptake time of 90 min. Patients were scanned immobilised in the radiotherapy treatment position to aid accurate image coregistration to the radiotherapy planning contrast-enhanced CT (CECT). The whole body from below the eyes to the mid-femurs was scanned. Scans were performed in 3D with a scan time of 4 min at each bed position. For the CT phase, 120 kV automA (max 250 mA), noise index 25.0 0.5 s/rotation, pitch 0.984:1, 3.75 mm slice width was used. Attenuation-corrected PET images were used in the analysis.



Figure 9: Timing of FDG-PET/CT in the ARC2 clinic trial. The timing of the FDG-PET/CT imaging in relation to the start and end of CRT was noted. The timing of the radiotherapy planning CECT is included as both CT components of the FDG-PET/CT were registered to it for region of interest definition.

2.2.3 Defining a region of interest for FDG-uptake quantification

The planning CT was loaded into Eclipse software (version 13.0, Varian Medical Systems, Palo Alto, CA, USA).¹⁹⁷ The GTV, as delineated by consensus of two radiation oncologists, was segmented on the exhale breath-hold CECT. The TV was noted. The process of transferring this 'tumour' ROI to the FDG-PET is summarised in Figure 10.

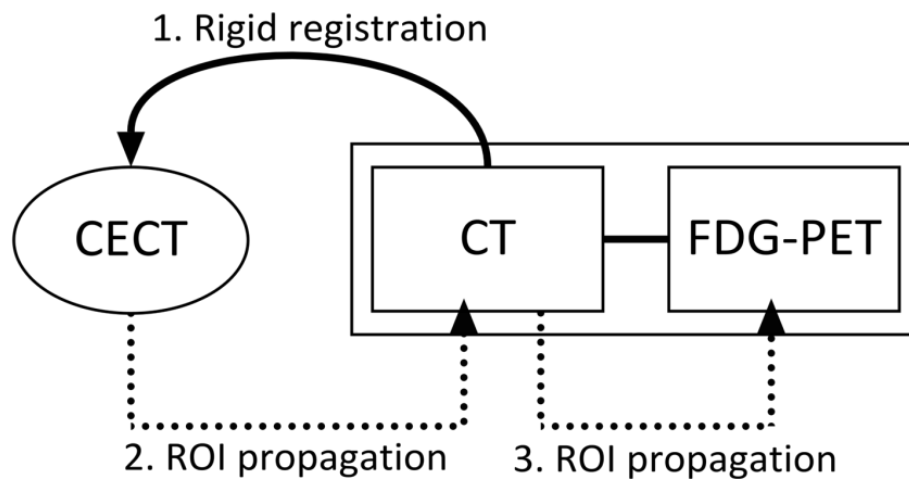


Figure 10: Obtaining a 'tumour' region of interest for quantification of the FDG-PET image. The planning CECT was used to outline the tumour. The CT component of the PET was then rigidly registered to the CECT and the tumour ROI was copied across. As the CT and PET of the FDG-PET/CT are hardware linked, the ROI could then be propagated on to the FDG-PET for uptake quantification.

The planning CECT was then rigidly registered to the CT component of the pre-CRT FDG-PET, prioritising accurate soft tissue matching in the region of the GTV. The GTV was propagated on to the CT component of the FDG-PET/CT to generate a 'tumour' ROI. As the PET and CT components of the PET/CT share the same frame of reference, the ROI could then be copied to the PET image. The same process was performed on the post-CRT FDG-PET/CT.

The extent of FDG uptake with the respect to the ROI was noted. Images were assessed to ensure that the FDG uptake could be attributed to tumour FDG retention. Any areas of uptake extending beyond the GTV were noted and an explanation of the FDG avidity was sought from the clinical information and imaging. As some reduction in tumour size could be seen on the CT component of the post-CRT FDG-PET/CT, some modification of the ROI was allowed on the post-CRT imaging. This was to exclude areas of non-tumour uptake (e.g. small bowel) contained within the tumour ROI that would artificially elevate FDG-PET parameters.

The PET image was then imported into PMOD software (a tool for PET quantitative assessment, Version 3.6, PMOD Technologies, Zurich, Switzerland).¹⁹⁸ FDG SUV_{max}, SUV_{peak} (the 1 cm³ volume within the ROI with the highest mean activity), SUV_{mean}, SUV_{median} and TLG were recorded for each image set. The percentage change in any parameter (%Δ) was calculated as follows:

$$\% \Delta = [(\text{Parameter}_{\text{post}} - \text{Parameter}_{\text{pre}}) / \text{Parameter}_{\text{pre}}] * 100$$

2.2.4 CA19-9 quantification

Serum was taken for CA19-9 quantification at baseline and again 6 weeks post CRT. This analysis was performed in a central laboratory using the ADVIA Centaur CA19-9 assay (Siemens Healthcare Diagnostics, Camberley, UK) as part of routine clinical care.

2.2.5 Patient follow-up

All patients had CECT of the chest, abdomen and pelvis every 3 months for 1 year. After 1 year, restaging CTs were performed at the discretion of the treating clinician. All post-CRT imaging was reviewed with a consultant radiologist. The timing and site of progression was noted. Imaging after the time of initial progression was also reviewed. For those who first progressed locally, the timing of development of metastatic disease was noted. For those who first progressed at a distant site, the presence or absence of local disease control was noted on each subsequent CECT. Patient follow-up was until the time of death or, for 3 surviving patients, until censoring on 28 January 2015.

2.2.6 Statistical analysis

Data was analysed in SPSS Statistics 22 (IBM, Portsmouth, UK).¹⁹⁹ Differences between groups were considered to be significant when $p < 0.05$. FDG-PET parameters and CA19-9 were compared before and after CRT using the Wilcoxon signed-ranks test. The correlation of SUV_{max} with the other PET-derived parameters at baseline and post CRT was characterised by Spearman's rho correlation coefficient and calculation of the p value of the correlation. Correlation of how all the PET parameters correlate with one another was investigated in the same way. Comparison of FDG-PET parameters was then performed during follow-up between patients with local disease (LD) only and those who developed

metastatic disease (MD), those who developed MD within 6 months of study entry and those who did not, and those who survived 1 year after trial entry and those who did not. Differences between these groups were sought by applying the Mann-Whitney U test. To see if any of the parameters correlate with OS, Spearman's rho correlation coefficient and the p value of the correlation were calculated. ROC curve analysis was performed to define the optimal cut-off of FDG-PET-derived parameters in predicting patients who only ever had LD during follow-up. The optimal cut-off was defined by the point on the ROC curve that minimised the number of false-positives and maintained identification of true-positives. Kaplan-Meier curves were generated for OS looking for differences in patients who achieved the recommended FDG-PET parameter cut-off for an improved OS (pre-CRT $SUV_{max} < 3.5$ or $\% \Delta SUV_{max} > 60\%$)⁴⁴ and those who did not. The patients were then divided based on whether pre- and post- SUV_{max} and $\% \Delta SUV_{max}$ were $<$ median or not and differences in OS using Kaplan-Meier analysis were sought. The significance of any difference in OS was tested with the log-rank test.

2.3 Results

2.3.1 Patient population

Of the 23 patients recruited to the study, 6 were not included in this subsequent analysis; 4 for clinical reasons and 2 for technical reasons. One patient died less than 2 months after trial entry because of pneumonia and did not reach the post-CRT FDG-PET/CT time-point. Three patients did not complete treatment as outlined in the protocol (1 biliary sepsis, 1 fatal pulmonary embolism, 1 stroke). This left 19 patients with sequential FDG-PETs for analysis. One patient was then excluded because of diffuse FDG uptake around a common bile duct stent that was continuous with, and could not be differentiated from, the

pancreatic tumour (see Figure 11). This was observed both pre- and post CRT. Another patient was excluded because of poorly controlled blood glucose (>20 mmol/L) at the time of scanning making image interpretation impossible. This left 17 patients (9 male, 8 female, mean age 65 years, range: 43–74) with sequential imaging that could be used in this analysis.

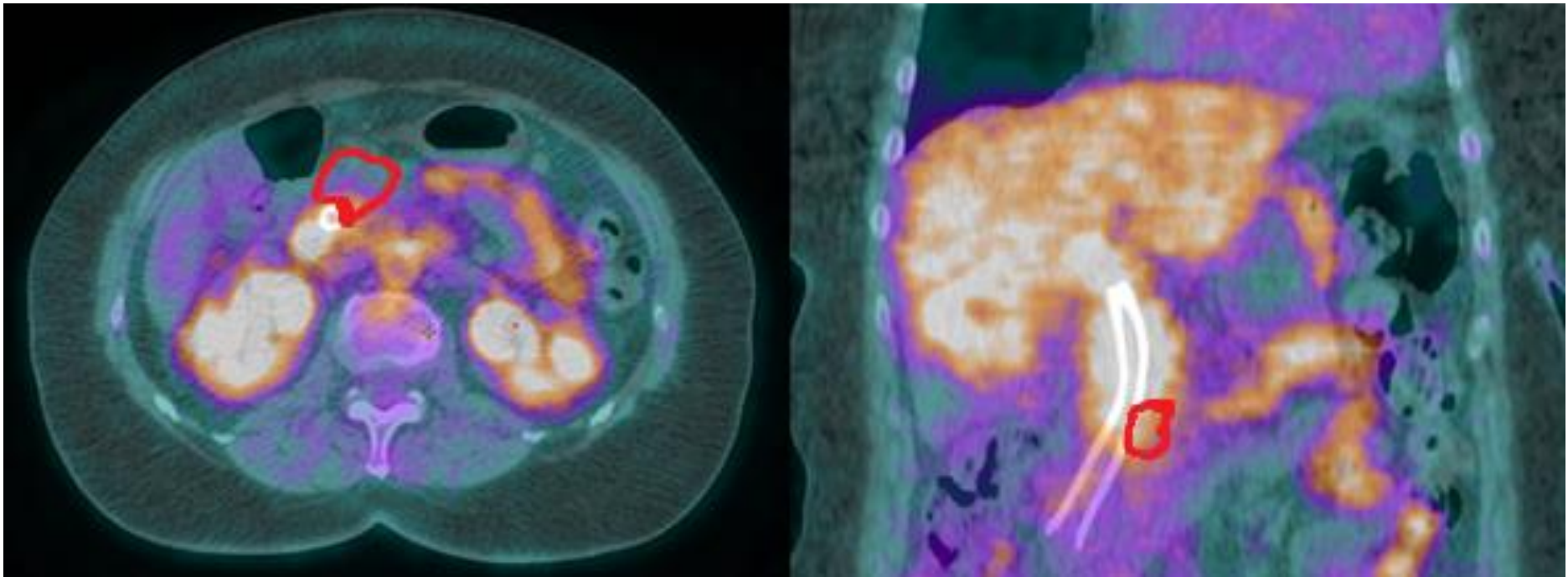


Figure 11: A transaxial and sagittal section from an FDG-PET/CT from the same patient. The tumour ROI is outlined in red. An area of uptake at the level of the tumour in the pancreatic head can be seen. This continues the full length of the metal stent in the common bile duct, with considerable spill-over of activity into the tumour ROI.

2.3.2 Timing of investigations

The pre-CRT FDG-PET was performed a mean of 22 days prior to study entry (range 14–34 days). The post CRT FDG-PET was a mean of 6.0 weeks (range 5.4–7.0 weeks) after the last day of CRT. CA19-9 data were available for all patients at baseline but were missing for two patients post CRT. The CA19-9 quantification was performed a mean of 14 days (range 0–61 days) before starting CRT and again a mean of 7.4 weeks (range 5.8–15.2 weeks) after completing CRT.

2.3.3 Changes in FDG-PET parameters and CA19-9

Changes in FDG-PET parameters and CA19-9 are summarised in Table 2. There was a significant reduction in SUV_{max} , SUV_{peak} , SUV_{mean} , SUV_{median} , TLG and CA19-9 from pre- to post CRT. A representative image of how FDG uptake changed from pre- to post CRT can be seen in Figure 12. All but one patient had a reduction in each of these values. One patient showed an increase in PET parameters. Interestingly, this same patient was the only person who also had an increase in CA19-9 from baseline to post CRT.

Table 2: Pre-CRT, post-CRT and relative change (%Δ) in FDG-PET-derived parameters and circulating CA19-9.

Parameter	Pre-CRT Median (range)	Post-CRT Median (range)	%Δ Median (range)	Significance p value (Wilcoxon signed-ranks test)
SUV_{max}	7.6 (2.6 to 15.6)	3.8 (1.6 to 7.9)	-44.2 (97.6 to -80.9)	0.002
SUV_{peak}	6.3 (2.0 to 13.5)	3.1 (1.6 to 6.1)	-41.4 (51.3 to -83.3)	0.001
SUV_{mean}	3.0 (1.6 to 5.6)	1.9 (1.1 to 3.3)	-35.1 (8.6 to -67.0)	<0.001
SUV_{median}	2.7 (1.6 to 5.2)	1.8 (1.1 to 2.8)	-33.5 (2.4 to -68.3)	<0.001
TLG	83.3 (16.8 to 208.0)	36.0 (8.8 to 167.5)	-46.5 (4.0 to -93.3)	<0.001
CA19-9 (U/mL)	441 (41 to 10,576)	60 (21 to 1,547)	-26.1 (26.1 to -90.3)	0.002

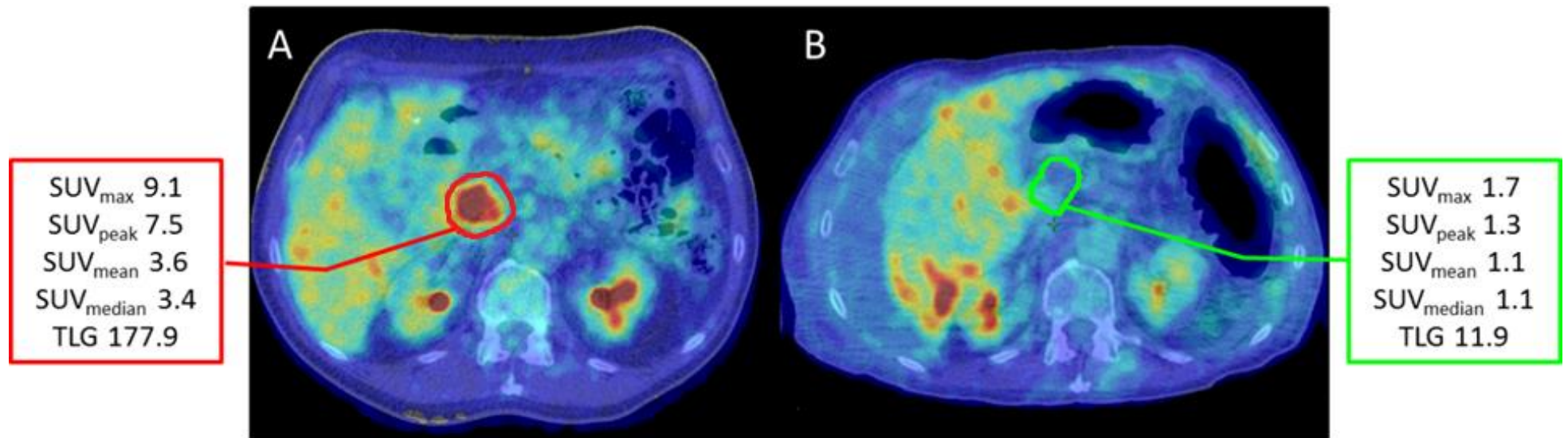


Figure 12: Transaxial images from FDG-PET/CTs in Patient 10 from the ARC2 study. Considerable changes can be seen from pre-CRT (A) and post CRT (B). FDG avidity has decreased with a reduction in all of the PET parameters. The pre-CRT tumour ROI (red) had to be modified on the post-CRT image (green). This is because the patient had developed ascites, which displaced the tumour and liver. In addition, there was evidence of tumour shrinkage on the CT component of the PET/CT.

2.3.4 *SUV_{max} correlates closely with other FDG-PET parameters*

Correlation of pre-CRT SUV_{max} with SUV_{peak}, SUV_{mean}, SUV_{median} and TLG can be seen in Figure 13. The closest correlation can be seen between pre-CRT SUV_{max} and SUV_{peak} (Spearman's rho = 0.980), although all parameters have a significant correlation with SUV_{max} despite the parameter values being significantly different ($p < 0.001$). Spearman's rho for the correlation of SUV_{max} with SUV_{mean}, SUV_{median} and TLG was 0.870, 0.794 and 0.792 respectively ($p < 0.001$ for all parameter correlations) pre-CRT.

A very similar pattern was seen in the post-CRT FDG-PET images (see Figure 14), with the closest correlation being observed between SUV_{max} and SUV_{peak}. Spearman's rho for the correlation of SUV_{max} with SUV_{peak}, SUV_{mean}, SUV_{median} and TLG was 0.966, 0.951, 0.877 and 0.833 respectively ($p < 0.001$ for all parameter correlations) post CRT. The weakest, yet still significant, correlation was seen between SUV_{max} and TLG at both time-points.

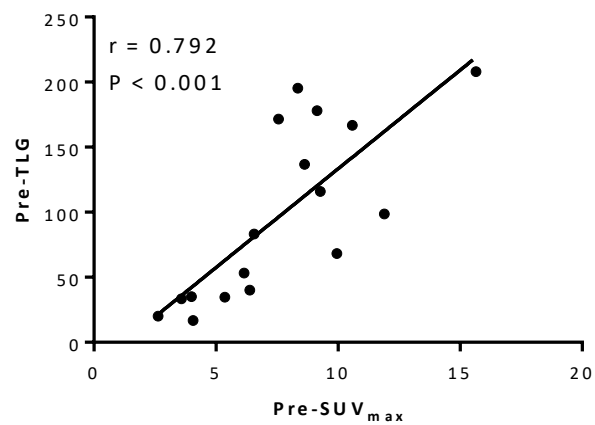
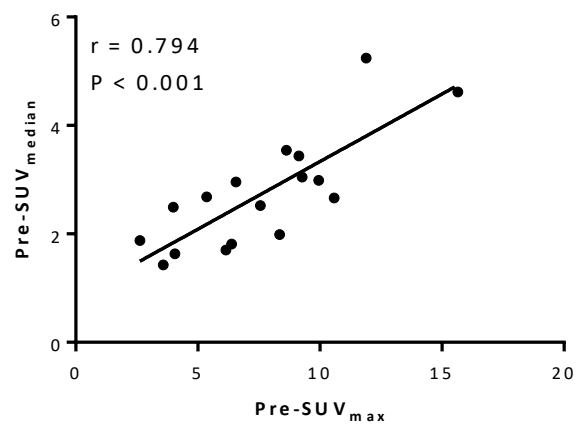
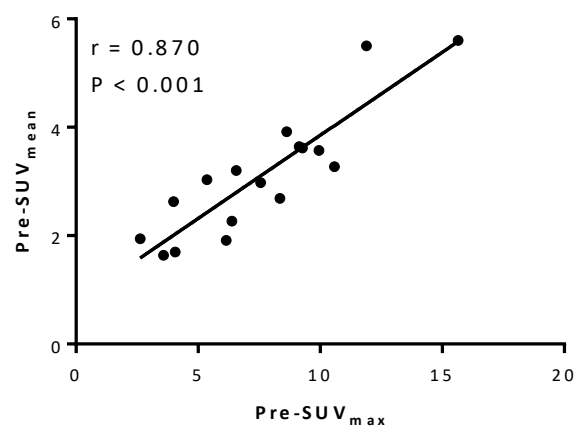
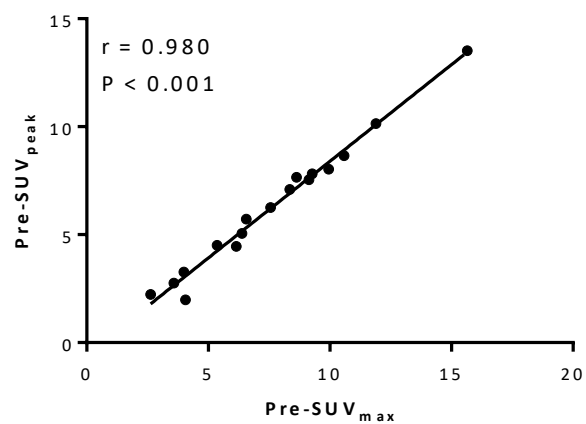


Figure 13: Scatterplots demonstrating the correlation of pre-CRT FDG-PET/CT SUV_{max} with SUV_{peak}, SUV_{mean}, SUV_{median} and TLG.

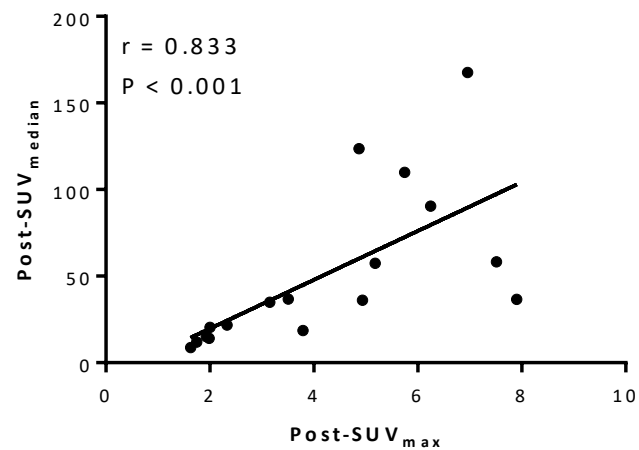
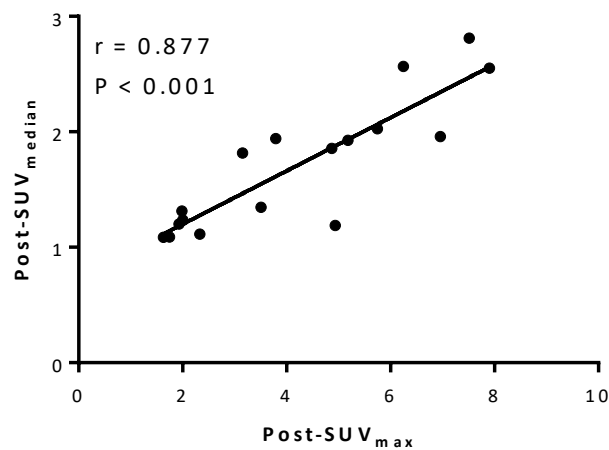
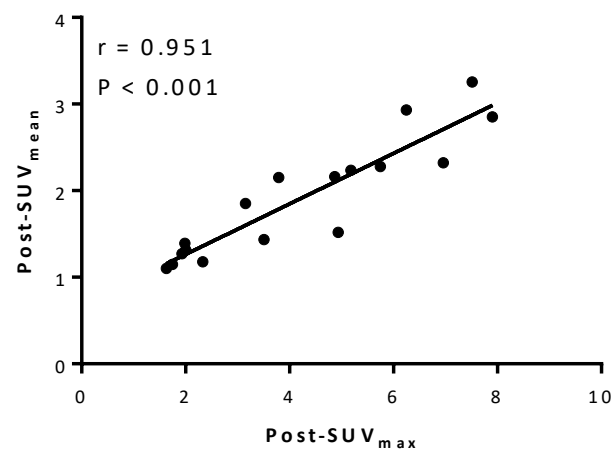
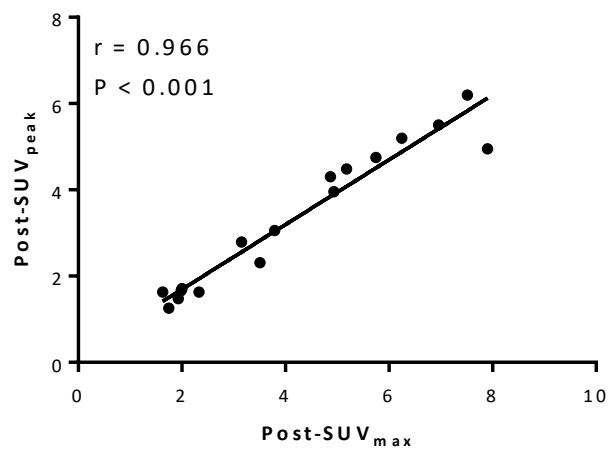


Figure 14: Scatterplots demonstrating the correlation of post-CRT FDG-PET/CT SUV_{max} with SUV_{peak}, SUV_{mean}, SUV_{median} and TLG.

2.3.5 *SUV_{peak}, SUV_{mean}, SUV_{median} and TLG are significantly correlated*

The relationship between the FDG-PET parameters that summarise FDG uptake over a ROI rather than reporting a single value can be seen when assessed pre-CRT (see Figure 15) and post CRT (Figure 16). As might be expected, the closest correlation observed was between SUV_{mean} and SUV_{median}, suggesting that the SUVs for each pixel within the ROI are approximately normally distributed. Again, the weakest, though still significant, correlations were seen between TLG (a parameter that incorporates TV) and the other parameters.

Pre-CRT, Spearman's rho for the correlation of SUV_{peak} with SUV_{mean}, SUV_{median} and TLG was 0.900, 0.831, 0.794 respectively (all $p < 0.001$). Spearman's rho for the correlation between SUV_{mean} with SUV_{median} and TLG were 0.985 ($p < 0.001$) and 0.620 ($p = 0.008$). SUV_{median} had a similar correlation with TLG with a Spearman's rho of 0.620 ($p = 0.008$).

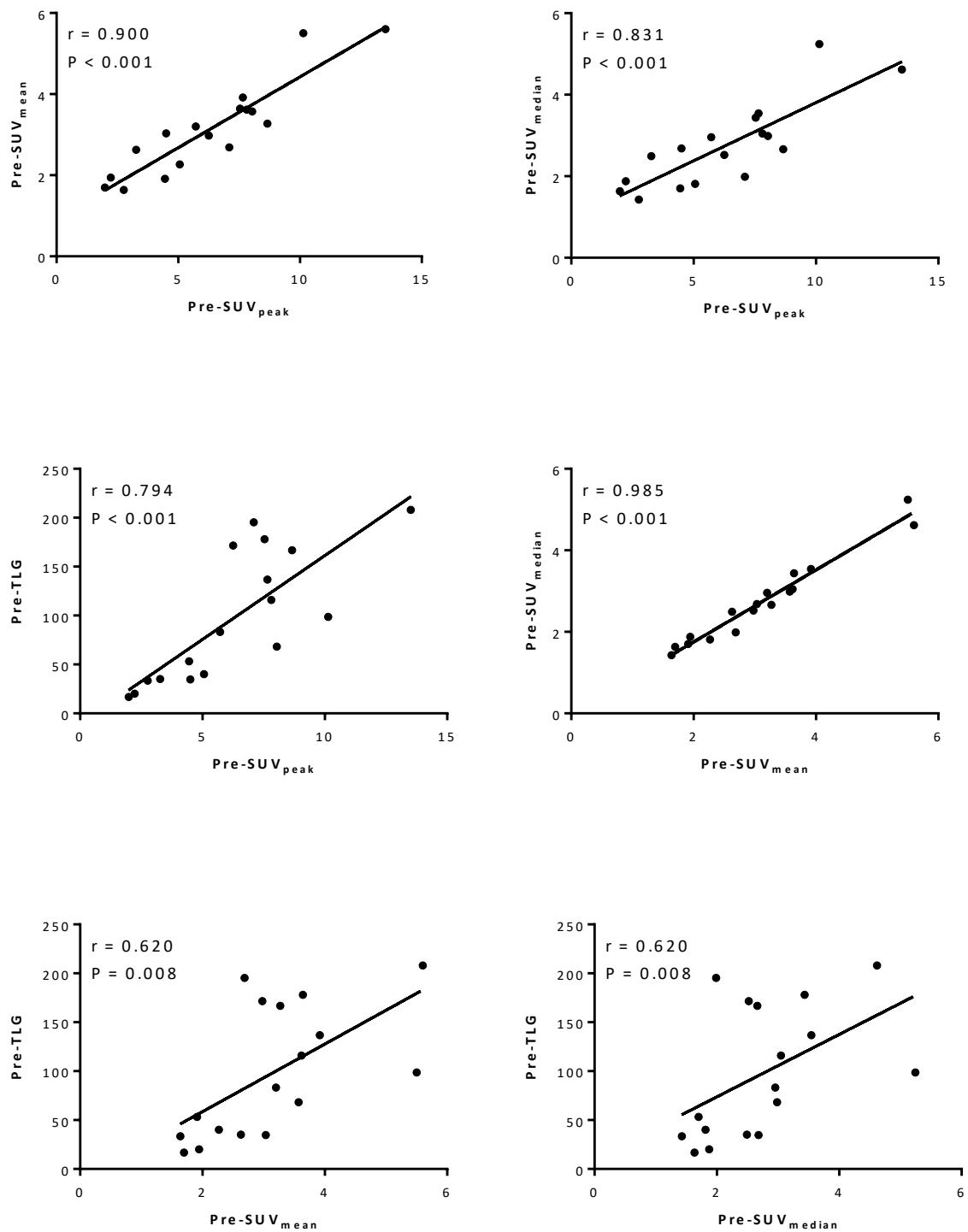


Figure 15: Scatterplots depicting the correlation of SUV_{peak}, SUV_{mean}, SUV_{median} and TLG with one another when assessed pre-CRT.

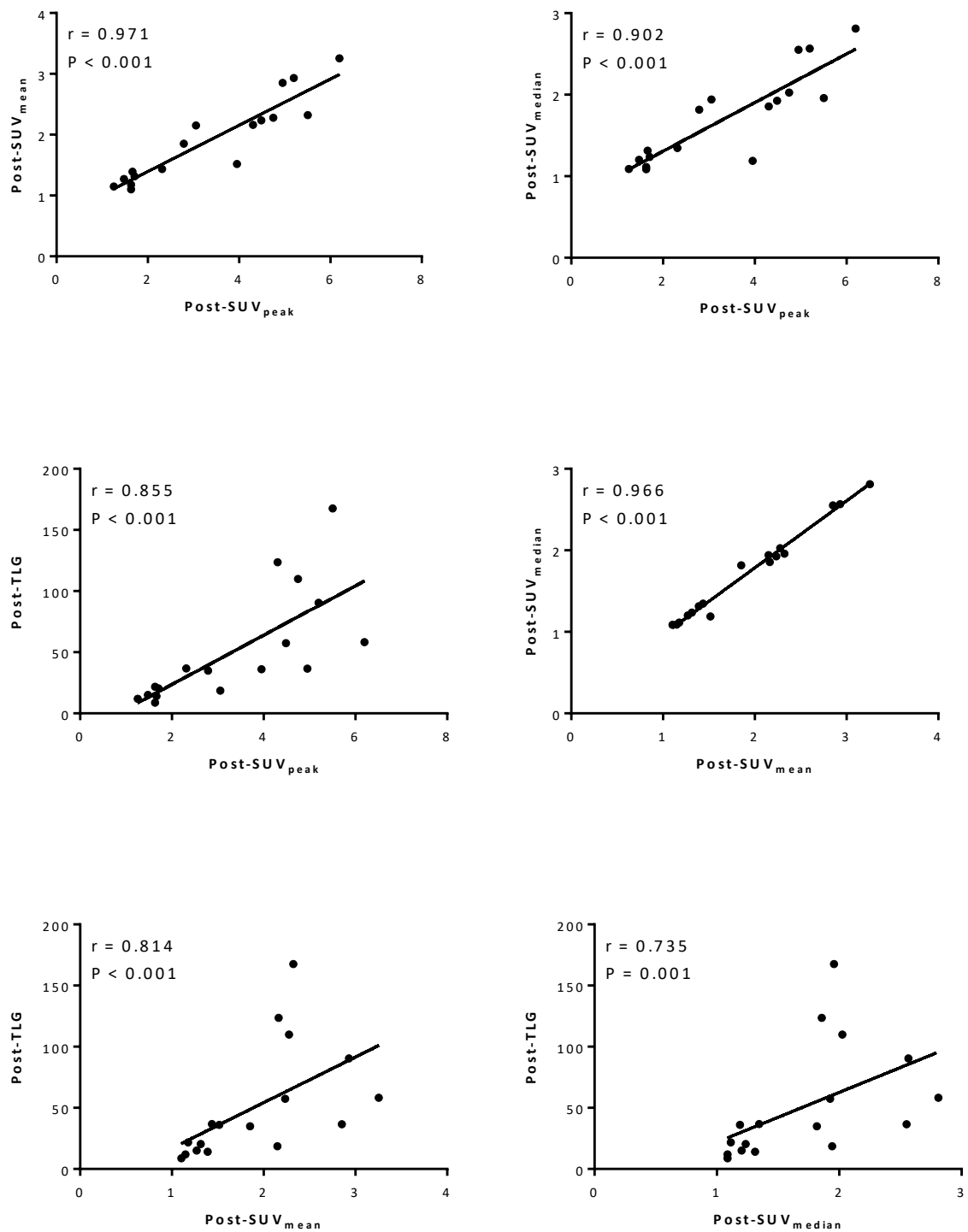


Figure 16: Scatterplots depicting the correlation of SUV_{peak}, SUV_{mean}, SUV_{median} and TLG with one another when assessed post CRT.

2.3.6 Univariate analysis to identify differences between patients with differing patterns of disease progression

2.3.6.1 Identifying patients who only experience local disease

From the 17 patients included in this analysis, 13 patients developed MD during follow-up. Four patients (24%) only ever experienced LD. Each of these 4 patients showed evidence of LD progression during the follow-up period. Median TV, pre- and post-CRT FDG-PET parameters and CA19-9 values for patients who in the LD-only group and those who developed MD can be seen in Table 3. There was no difference in pre-CRT TV between these groups. Apart from post-CRT SUV_{max} , there was no significant difference in the post-CRT or % Δ in PET parameters or CA19-9 between those who did not develop MD during follow-up and those who did. A statistically significant difference was observed in the pre-CRT FDG-PET parameters between those who had LD only and those who went on to develop MD. Pre-CRT FDG-PET parameters that averaged uptake activity across the whole tumour ROI were clearly distinct between the two groups. TLG, which combines SUV_{mean} with TV, weakened the difference between the two groups that had been observed with SUV_{mean} alone.

A planned multivariate analysis was not performed as the LD-only group contained only 4 patients. A predictive model would be unlikely to correctly identify only 4 out of 17 patients.

Table 3: Comparison of pre- and post-CRT and relative change in FDG-PET-derived parameters and CA19-9 in patients who never developed distant disease during follow-up (local pancreatic disease only) and those who did develop MD.

Parameter	LD only during follow-up Median (range) n = 4	Developed distant disease during follow-up Median (range)	Significance p value (Mann- Whitney U test)
Baseline TV (cm ³)	21.6 (1.6–38.0)	32.9 (11.3–80.2)	0.202
Pre-SUV _{max}	3.8 (2.6–6.2)	8.6 (4.0–15.6)	0.006
Pre-SUV _{peak}	2.5 (2.0–4.5)	7.5 (4.5–13.5)	0.002
Pre-SUV _{mean}	1.8 (1.6–1.9)	3.3 (2.2–5.6)	0.001
Pre-SUV _{median}	1.7 (1.4–1.9)	3.0 (2.5–5.2)	0.002
Pre-TLG	26.9 (16.8–53.3)	115.9 (35.2–208.8)	0.006
Pre-CA19-9 (U/mL)	509 (135–576)	387 (35–10,576)	0.785
Post-SUV _{max}	2.0 (1.6–3.5)	4.9 (1.7–7.9)	0.045
Post-SUV _{peak}	1.7 (1.6–2.3)	4.3 (1.3–6.2)	0.102
Post-SUV _{mean}	1.4 (1.1–1.4)	2.2 (1.1–3.3)	0.060
Post-SUV _{median}	1.3 (1.1–1.3)	1.9 (1.1–2.8)	0.130
Post-TLG	17.2 (8.8–36.7)	36.6 (15.1–167.5)	0.102
Post-CA19-9 (U/mL)	93 (36–149)	30 (21–1547)	0.661
%ΔSUV _{max}	–43.6 (–24.5––59.8)	–45.7 (97.5––80.9)	0.785
%ΔSUV _{peak}	–31.7 (–17.8––48.1)	–45.2 (51.2––83.3)	0.384
%ΔSUV _{mean}	–27.0 (–19.8––35.1)	–42.2 (8.6––67.0)	0.130
%ΔSUV _{median}	–25.4 (–13.6––30.0)	–45.2 (51.3––83.3)	0.202
%ΔTLG (%)	–35.2 (–30.0––47.7)	–56.5 (4.0––93.0)	0.296
%ΔCA19-9 (U/mL)	–82.0 (–7.4––91.8)	–59.5 (82.1––99.8)	0.949

2.3.6.2 Identifying patients who develop early metastatic disease

Table 4 contains median pre-, post-CRT and %Δ in FDG-PET parameters and CA19-9 in those who developed MD in less than 6 months. There was no statistically significant difference in these parameters in those who developed MD compared with those who did not.

A planned multivariate analysis was not performed as none of the parameters was statistically significant in this univariate analysis.

Table 4: Comparison of pre- and post-CRT and relative change in FDG-PET-derived parameters in patients developing MD in less than 6 months from trial entry with those who did not.

Parameter	Developed MD in <6 months post-study entry Median (range) n = 4	Did not develop MD in <6 months post-study entry Median (range)	Significance p value (Mann-Whitney U test)
Baseline TV (cm ³)	34.6 (11.3–56.2)	28.6 (10.6–80.2)	0.956
Pre-SUV _{max}	8.4 (5.4–10.0)	6.6 (2.6–15.6)	0.703
Pre-SUV _{peak}	6.9 (4.5–8.0)	5.7 (1.9–13.5)	0.703
Pre-SUV _{mean}	3.3 (3.0–3.6)	2.7 (1.7–5.6)	0.477
Pre-SUV _{median}	2.8 (2.5–3.4)	2.5 (1.6–5.2)	0.412
Pre-TLG	119.9 (34.7–177.9)	83.3 (16.8–208.0)	0.549
Pre-CA19-9 (U/mL)	265 (41–2723)	441 (35–10,576)	0.477
Post-SUV _{max}	3.1 (1.7–3.8)	4.9 (1.6–7.0)	0.350
Post-SUV _{peak}	2.3 (1.3–4.3)	4.0 (2.8–6.2)	0.245
Post-SUV _{mean}	1.6 (1.1–2.2)	1.9 (1.1–3.3)	0.350
Post-SUV _{median}	1.5 (1.1–1.9)	1.8 (1.1–2.8)	0.350
Post-TLG	20.2 (11.9–123.5)	36.6 (15.1–167.5)	0.412
Post-CA19-9 (U/mL)	30 (22–1284)	91 (21–1574)	0.840
Δ SUV _{max} (%)	–56.1 (–29.2– –80.9)	–44.2 (97.6– –69.7)	0.549
Δ SUV _{peak} (%)	–55.9 (–31.1– –83.3)	–41.4 (51.3– –70.8)	0.412
Δ SUV _{mean} (%)	–48.1 (–27.3– –68.4)	–35.1 (8.6– –58.1)	0.271
Δ SUV _{median} (%)	–45.2 (–26.3– –68.3)	–33.5 (2.4– –61.0)	0.254
Δ TLG (%)	–57.2 (–28.0– –93.3)	–40.9 (4.0– –62.3)	0.310
Δ CA19-9 (U/mL)	–46.3 (–18.9– –52.8)	–82.0 (82.7– –99.8)	0.180

2.3.6.3 ROC curve analysis for patients who did not develop metastatic disease during follow-up

ROC curve analysis for all of the FDG-PET parameters can be seen in Table 5. ROC curve analysis found pre-CRT SUV_{mean} to be the best predictor of those (n = 4) who never experience MD. The more easily accessible parameter, SUV_{max}, performed well when quantified pre-CRT (AUC = 0.932). The pre-CRT parameters appear to be more predictive of patients who only ever have LD rather than progressing distally than post-CRT parameters.

Table 5: ROC curve analysis of the ability of pre- and post CRT FDG-PET to predict patients who will only ever have local disease (no metastatic spread). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) are presented.

Parameter	ROC Curve AUC	95% CI	Parameter cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Pre-SUV_{max}	0.932	0.802–1.000	6.2	100.0	92.3	80.0	100.0
Pre-SUV_{peak}	0.977	0.909–1.000	4.5	100.0	92.3	80.0	100.0
Pre-SUV_{mean}	1.000	1.000–1.000	2.1	100.0	100.0	100.0	100.0
Pre-SUV_{median}	0.977	0.909–1.000	1.9	100.0	92.3	80.0	100.0
Pre-TLG	0.932	0.787–1.000	60.7	100.0	76.9	57.1	100.0
Post-SUV_{max}	0.886	0.712–1.000	3.7	100.0	69.2	50.0	100.0
Post-SUV_{peak}	0.841	0.632–1.000	2.5	100.0	76.9	57.1	100.0
Post-SUV_{mean}	0.864	0.670–1.000	1.5	100.0	76.9	57.1	100.0
Post-SUV_{median}	0.795	0.560–1.000	1.6	100.0	69.2	50.0	100.0
Post-TLG	0.818	0.562–1.000	47.1	100.0	46.2	36.4	100.0

2.3.7 Correlation of FDG-PET parameters and CA19-9 with survival

The primary end-point of the ARC2 clinical trial was 1-year OS. Univariate analysis did not identify any statistical difference in the FDG-PET parameters or CA19-9 in those who achieved 1-year OS (n = 14) and those who did not (n = 3). A statistically significant difference was observed in the relative change in TLG for those who reached 1-year survival and those who did not (–40.1% vs –56.5%, p = 0.023). No significant correlation was observed between any of the other tested variables and OS (see Table 6).

Table 6: Comparison of pre- and post-CRT and relative change in FDG-PET-derived parameters in patients surviving 1 year and those who did not survive for 1 year after study entry. Spearman's rho correlation coefficient of the association of the parameter with OS is also presented.

Parameter	Achieved 1-year survival Median (range)	Did not achieve 1- year survival Median (range) n = 3	Significance p value (Mann-Whitney U test)	Spearman's rho correlation coefficient (with OS)	p value of correlation with OS
Baseline TV (cm³)	27.9 (10.6–80.2)	44.9 (11.3–49.9)	0.676	–0.088	0.736
Pre-SUV_{max}	7.1 (3.5–11.9)	9.1 (5.4–15.6)	0.432	–0.266	0.302
Pre-SUV_{peak}	6.0 (1.9–10.1)	7.5 (4.5–13.5)	0.432	–0.279	0.315
Pre-SUV_{mean}	2.8 (1.6–5.5)	3.6 (3.0–5.6)	0.121	–0.129	0.648
Pre-SUV_{median}	2.5 (1.4–5.2)	3.4 (2.7–4.6)	0.121	–0.466	0.059
Pre-TLG	75.7 (16.8–171.5)	177.9 (34.7–208.0)	0.300	–0.393	0.147
Pre-CA19-9 (U/mL)	509 (35–10,576)	78 (41–489)	0.048	0.313	0.222
Post-SUV_{max}	4.2 (1.6–7.5)	3.8 (1.7–6.2)	0.859	0.013	0.959
Post-SUV_{peak}	3.4 (1.6–6.2)	3.1 (1.3–5.2)	0.859	0.037	0.889
Post-SUV_{mean}	1.7 (1.1–3.3)	2.2 (1.1–2.9)	0.953	–0.264	0.341
Post-SUV_{median}	1.6 (1.1–2.8)	1.9 (1.1–2.6)	0.707	–0.071	0.786
Post-TLG	36.3 (8.8–167.5)	18.6 (11.9–90.4)	0.509	0.128	0.626
Post-CA19-9 (U/mL)	91 (21–1547)	22 (22)	0.606	0.199	0.478
ΔSUV_{max} (%)	–43.6 (97.6– –68.0)	–60.1 (–29.2– 80.9)	0.362	0.391	0.121
ΔSUV_{peak} (%)	–40.1 (51.3– –79.7)	–61.5 (–32.2– 83.3)	0.244	0.114	0.685
ΔSUV_{mean} (%)	–32.7 (8.6– –67.0)	–47.7 (–29.1– 47.7)	0.197	0.430	0.085
ΔSUV_{median} (%)	–31.7 (2.4– –68.3)	–44.4 (–27.6– 68.3)	0.300	–0.007	0.980
ΔTLG (%)	–40.1 (3.9– –68.0)	–56.5 (–46.4– 93.3)	0.244	0.546	0.023
ΔCA19-9 (U/mL)	–71.5 (82.1– –99.8)	–46.3 (–46.3)	0.844	0.100	0.723

2.3.8 The prognostic utility of pre-, post-CRT and relative change in FDG-PET

SUV_{max}

Median OS in the 17 patients included in this analysis was 18.1 months (95% CI 16.3–19.9 months). When the patients were divided into groups that contained patients who had a pre-CRT SUV_{max} < 3.5 or %ΔSUV_{max} > 60% and those who did not, as recommended

by Chang *et al.*⁴⁴, only 4 patients met the described criteria. No difference in median OS was observed between the two groups (18.1 vs 9.1 months with 95% CIs of 16.3–20.0 and 0.0–32.7 months respectively, $p = 0.669$). Kaplan-Meier curves for pre-CRT, post-CRT and $\% \Delta$ in SUV_{max} using the population median to separate the patients into two groups can be seen in Figure 17. As hypothesised, single time-point SUV_{max} did not offer any prognostic information when the patients were divided into two groups based on the population median. There was no significant difference in OS in those with a pre-CRT $SUV_{max} < 7.6$ (18.1 vs 18.9 months, $p = 0.816$) or post-CRT $SUV_{max} < 3.8$ (14.6 vs 18.8 months, $p = 0.816$). The median relative change in SUV_{max} from pre- to post CRT was 44.2%. Patients with a $>44.2\%$ decrease in SUV_{max} had a median OS of 23.0 months (95% CI 7.3–39.1 months) compared with 14.6 months (95% CI 16.3–19.9 months) ($p = 0.013$).

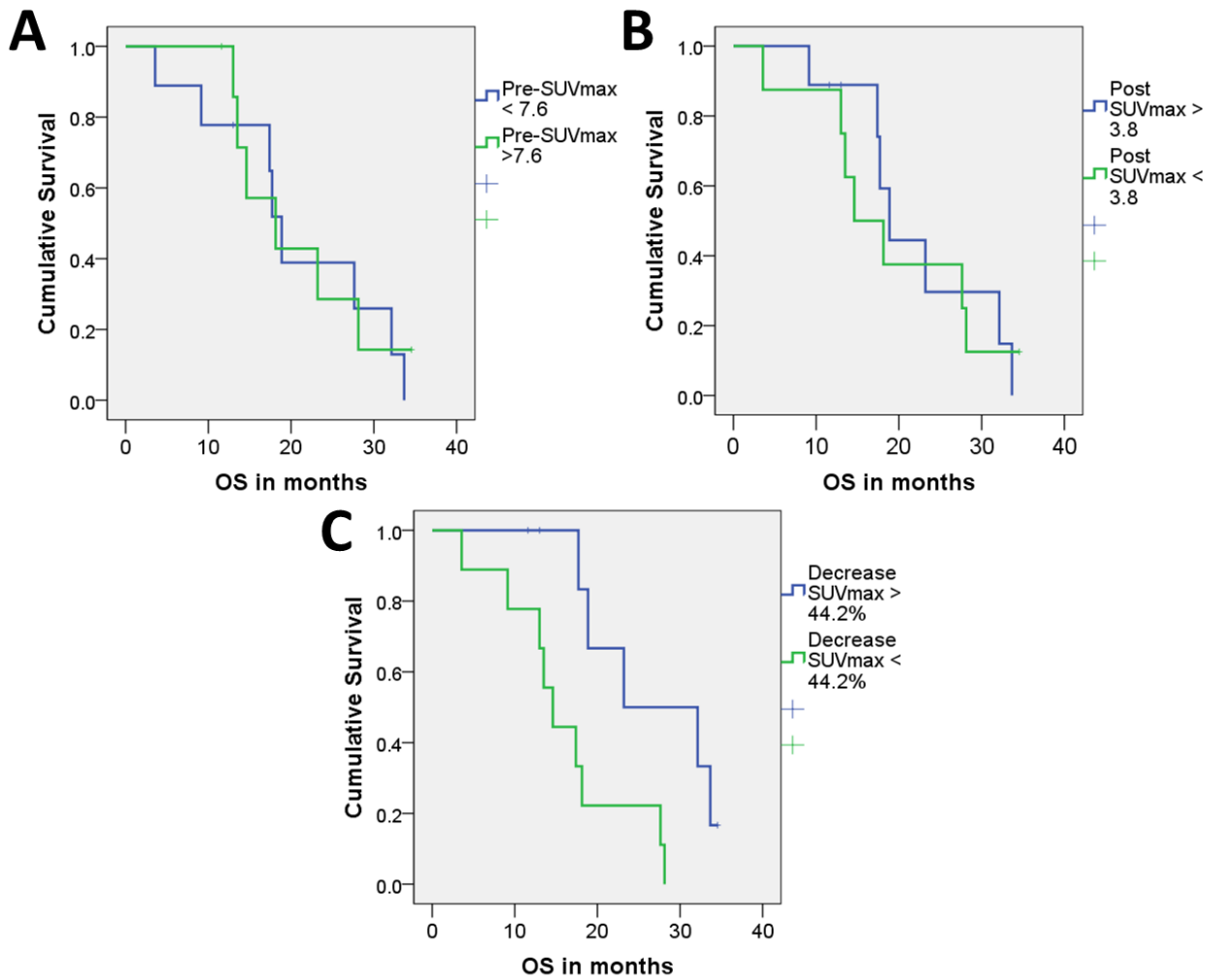


Figure 17: Kaplan-Meier curves depicting OS based on pre-CRT (A), post-CRT (B) and relative change in SUV_{max} being greater or less than the population median (C).

2.4 Discussion

LAPC has a poor prognosis and recent evidence has suggested that CRT does not improve survival compared with chemotherapy alone.²⁰ As we know that some patients only ever experience disease at the site of the pancreatic tumour, it would seem that there should be a role for upfront locally intensive treatment in a subgroup of patients – if only this group could be accurately identified at the point of diagnosis. A greater understanding of patient prognosis, based on an individual assessment, would be beneficial in guiding post-CRT treatment decisions and in being able to provide realistic, personalised information to

patients. Our findings suggest that the inclusion of FDG-PET at the point of diagnosis may help guide treatment strategy selection and that an FDG-PET response assessment may inform about patient prognosis following upfront CRT. The best means of identifying the most useful FDG-PET parameters requires further investigation.

The derivation of FDG-PET/CT-derived parameters is not a process that can be automated and the imaging analysis should always take the clinical context and pattern of FDG-uptake into account. While it would have been possible to obtain parameter values for two patients who were excluded from this analysis (one because of elevated serum glucose at the time of scanning and one because of inflammation around a common bile duct stent), the activity that is being quantified does not relate to tumour glucose metabolism and could therefore be misleading.

The impact that variability in timing of the imaging tests in relation to the start of CRT has on the test's predictive or prognostic utility is uncertain. While efforts were made to limit the variability in the number of days from FDG-PET scanning to starting CRT, the mean time pre-CRT that the FDG-PET was performed in this series was 22 days, with a range of 14–34 days. This variability should be taken in to account when analysing a larger cohort. The potential for FDG-PET progression over 14–34 days in LAPC is not known, but could affect the predictive and prognostic function of the test.

The median value of all of the assessed variables, FDG-PET parameters and CA19-9, decreased following CRT. A marked heterogeneity in response was seen. For example, the $\% \Delta \text{SUV}_{\text{max}}$ varied from an increase of 97.6% to a decrease of 80.9%. This heterogeneity may explain the usefulness of the $\% \Delta \text{SUV}_{\text{max}}$ parameter in informing about patient prognosis in this cohort of patients.

While SUV_{max} correlates very closely with SUV_{peak} , SUV_{mean} and SUV_{median} , the correlation with TLG is significant, but less closely correlated than with the other parameters. This is, perhaps, unsurprising given that TLG factors in TV. It is known that pancreatic tumours have a large stromal component. The stroma, composed of fibroblasts, pancreatic stellate cells, immune cells and extracellular matrix proteins such as types I and III collagens,²⁰⁰ contributes to the volume of the tumour, but may in comparison to the tumour cells be relatively metabolically quiescent. This observation may also explain the failure of TV to predict patients with only LD when other pre-CRT variables did differ in this group. It is not the size of pancreatic tumours that informs about behaviour, but rather the metabolic activity within this volume.

The SUV_{peak} is the activity in the 1 cm^3 sphere that has the highest mean activity within the ROI. It was thought that using the SUV_{peak} rather than SUV_{max} would reduce some of the uncertainty associated with the SUV_{max} because of the effect of noise on this single-pixel parameter. The observed results suggest that SUV_{max} and SUV_{peak} correlate extremely closely (Spearman's $\rho = 0.980$) in LAPC, despite the numerical values being significantly different. This is reassuring as the SUV_{max} is the most commonly used PET parameter used in response prediction studies and is often routinely available in routine clinical practice. This finding needs to be replicated in a larger cohort of patients before the detailed analysis of uptake values across the whole ROI are no longer taken into account. Calculation of SUV_{peak} , SUV_{mean} and SUV_{median} is more labour intensive than simply obtaining the maximum value, but appears to be worthwhile in other tumour sites.^{69,70,201} Although this series is too small to demonstrate any real difference in the ability of the parameters to identify patients with only LD, there is a suggestion that SUV_{peak} , SUV_{mean} and SUV_{median} may

be more robust than SUV_{max} and TLG given the smaller p values and greater ROC curve AUC.

Once patients who did not complete therapy and those with incomplete or uninterpretable imaging were excluded, only 17 patients were able to be included in this analysis. This small cohort is probably a reflection of pancreatic cancer being a relatively rare condition. Finding an additional cohort of patients to combine with this group was not possible. FDG-PET staging and response assessment is not currently routine in LAPC. Patients recruited to recently published series SCALOP and LAP07 did not routinely have FDG-PET studies.^{19,20} All of our observations and recommendations are therefore presented with a degree of caution as they are drawn from such a small sample.

The finding that pre-CRT FDG-PET parameters tend to be lower in those patients who experience only LD and did not develop MD during follow-up shows promise. Obtaining these values in a larger cohort of patients with LAPC treated with upfront CRT may identify which parameters are most useful in identifying patients who only ever have LD and may therefore benefit from upfront CRT, potentially with local treatment intensification. Our sample size excluded a multivariate analysis to identify the most useful parameters, particularly as only 4 patients were in the LD group. These findings are particularly promising as pre-CRT assessment would help in treatment strategy selection. Validation of our findings in a larger cohort could lead to a clinical trial in which local treatment intensification may be offered to those with a high likelihood of having only LD. It may also inform the sequencing of therapies, with an ability to recommend upfront (chemo)radiotherapy. There is a trend in recent studies in LAPC^{19,20} to offer induction chemotherapy followed by CRT in those whose disease does not progress outside of the

pancreas. Achieving good local control early in the treatment schedule may translate into a clinical benefit in patients in the LD group.

A difference in FDG-PET parameters or CA19-9 was not observed between patients who developed early MD and those who did not. A greater understanding of the patients who develop MD soon after definitive CRT is required. It could be that more intensive attempts at achieving local control may prevent the development of MD. Accurate staging to identify patients who already have MD is an essential first step in identifying a subgroup of patients for upfront CRT and radiotherapy dose escalation. FDG-PET appears to have a role as its sensitivity for detecting low volume MD is higher than that of conventional CT imaging.^{44,202} Seventeen percent of patients screened for entry into this study were excluded because of the identification of previously unrecognised MD on the screening FDG-PET scan.²⁰³ Despite this, FDG-PET/CT is not currently part of the routine staging investigations for LAPC in the UK.

The only parameter to show a difference in patients who attained 1-year survival was the relative change in TLG. This observation was not investigated further as the observed statistical significance may be a result of multiple testing on a very small cohort of patients (only 3 patients failed to survive 1-year post-study entry). Seeing if differences in TLG between patients who survive 1 year and those who do not remains statistically significant in a multivariate analysis would require a larger cohort of patients.

The hypothesis that the relative change in SUV_{max} would offer more prognostic differentiation than the absolute SUV_{max} when measured pre- or post CRT was upheld and the null hypothesis that no FDG-PET parameter is prognostic can be rejected. There was an increase in median OS in patients whose $\% \Delta SUV_{max}$ was greater than the median for this

cohort. These data were not able to validate the use of a pre-CRT SUVmax < 3.5 or $\Delta\text{SUV}_{\text{max}} > 60\%$ to predict OS as suggested by Chang *et al.*⁴⁴ This could be because of the heterogeneity in treatment regimens and variability in the timing of imaging in the published series. It could also be because this cohort of only 17 patients was too small. While this prognostic information obtained from $\Delta\text{SUV}_{\text{max}}$, if confirmed in a larger series, may be helpful when counselling patients about their treatment response, it relies on a post-CRT assessment. It would therefore not be helpful in treatment sequencing. It may, however, be useful in guiding adjuvant therapy decisions. For example, patients with minimal $\Delta\text{SUV}_{\text{max}}$ may be offered more intensive adjuvant chemotherapy regimens to counteract the potentially poorer OS. For example, there is now evidence that the intensive chemotherapy regimens FOLFIRINOX and gemcitabine in combination with nab-paclitaxel prolong survival in metastatic pancreatic cancer.^{204,205} Failure of a metabolic response as assessed by FDG-PET may therefore select a population of patients who should be offered these intensive regimens early post CRT in the adjuvant setting in the hope of delaying the time to the development of MD. It may not be appropriate to offer all patients these treatments because of their associated toxicities, but these toxicities may be acceptable to patients with a high risk of development of disease outside of the pancreas. This could be tested in a clinical trial.

The serum CA19-9 level has been shown to be prognostic in the postoperative setting²⁰⁶ and it has been suggested that the combination of a pretreatment CA19-9 > 1,200 U/mL, post-treatment CA19-9 > 100 U/mL and a decline in CA19-9 of $\leq 40\%$ has been associated with poor survival following definitive CRT for pancreatic cancer.²⁰⁷ A wide range of CA19-9 levels was observed in this series. Values ranging from 35 to 10,576 U/mL were seen pre-

CRT and values of 22 to 1,574 U/mL were seen post CRT. These values are in keeping with published series. In the preoperative setting, CA19-9 values ranging from 1 to 26,600 U/mL (median 138 U/mL) have been reported.²⁰⁶ Similarly, in the pre-CRT setting, CA19-9 levels ranged from 48 to 20,000 U/mL and were seen falling to a range of values from 6 to 7,350 U/mL post CRT.²⁰⁷ The CA19-9 levels seen here were in the range that might have been expected and a fall in CA19-9 was seen post CRT. The pre- or post-CRT, or decline in, CA19-9 levels in this series were not predictive of the pattern of disease progression and could therefore not be used to identify a group of patients in whom local treatment intensification might prolong survival.

The observation that patients with low pre-CRT FDG uptake (thresholds identified by ROC curve analysis: $SUV_{max} < 6.2$, $SUV_{peak} < 4.5$, $SUV_{mean} < 2.1$, $SUV_{median} < 1.9$) appear to never develop MD potentially identifies a patient group who may benefit from upfront CRT, potentially with dose escalation of the radiotherapy to achieve lasting local control. Previous attempts at radiotherapy dose escalation in LAPC have been limited by normal tissue toxicity – most notably to the duodenum.³⁸ The next chapter will investigate how functional imaging may be used to identify smaller subregions of the tumour that may be suitable targets for dose escalation.

2.5 Conclusions

SUV_{max} is easy to obtain from FDG-PET images and is the most commonly used parameter in response prediction studies. Although this single-pixel value is subject to noise, it was significantly correlated with SUV_{mean} , SUV_{median} , SUV_{peak} and TLG in this cohort of patients with LAPC when assessed both pre- and post CRT. This observation supports the ongoing use of SUV_{max} in FDG-PET studies in LAPC, and the null hypothesis that there is no

correlation between SUV_{max} and other FDG-PET parameters can be rejected. The null hypothesis that SUV_{mean} , SUV_{median} , SUV_{peak} and TLG are not significantly correlated with one another can also be rejected, suggesting that information is common to each of the FDG-PET parameters. It may be that future studies that aim to assess the predictive and prognostic utility of FDG-PET in CRT-treated LAPC do not need to calculate all of these parameter values.

Pre-CRT FDG-PET parameters can identify patients who will only ever have cancer localised to the pancreas. These findings suggest that patients with less FDG-avid tumours are less likely to develop MD and may therefore benefit from upfront local treatment intensification. The null hypothesis that there is no relationship between FDG-PET-derived parameters or CA19-9 and patterns of disease progression following CRT can be rejected in so far as FDG-PET parameters identify patients who do not develop MD during follow-up, but it cannot be rejected for the prediction of early MD. A relative response in SUV_{max} was more informative about OS than absolute values pre- and post CRT. In this cohort of patients, a $\% \Delta SUV_{max} > \text{median}$ for this population (42%) was associated with prolonged OS.

The application of FDG-PET to treatment strategy selection and prognostication in LAPC therefore shows promise, but these findings require testing and validation in a larger cohort of patients.

Chapter 3: Using functional imaging to derive a radiotherapy biological target volume; moving towards hypoxia targeting and modulation

3.1 Introduction

The aims of the work in this chapter are:

- To see if regions of high FDG uptake within the pancreatic tumour prior to CRT may identify areas of residual FDG avidity post CRT.

This has been suggested to be the case in NSCLC⁹⁹ and rectal cancer.¹⁰⁰ If residual FDG avidity 6 weeks after CRT for LAPC was spatially related to high FDG uptake on a baseline FDG-PET scan, this area could be thought of as 'radioresistant' and may serve as a means of delineating a BTV that could be dose escalated. The null hypothesis to be tested states that there is no spatial relationship between areas of tumour FDG uptake pre-CRT with areas of residual FDG avidity post CRT.

- To assess the utility of FMISO-PET as a means of characterising hypoxia in LAPC.

For FMISO-PET to be used to define a BTV for radiotherapy dose escalation, there would need to be evidence of FMISO uptake and the region of peak hypoxia would need to be spatially consistent. The null hypothesis to be tested states that there is no spatial relationship between areas of FMISO uptake within pancreatic tumours on repeat testing.

- To test whether there is a change in tumour hypoxia as a result of pAKT inhibition using nelfinavir therapy in LAPC using FMISO-PET.

This change may be seen on uptake volumes seen 4 h post FMISO injection or on changes in kinetic parameter values associated with FMISO retention on dynamic PET imaging. The null hypothesis to be tested states that nelfinavir has no effect on tumour FMISO-retention.

- To characterise any changes in tumour perfusion as a result of nelfinavir therapy using pCT.

The use of pCT has been investigated previously in pancreatic cancer.²⁰⁸⁻²¹⁰ Here, it was performed at the same time-points as the FMISO-PET to answer a secondary question as to whether nelfinavir modulates hypoxia by increasing tumour perfusion. The structure of pancreatic tumours is complex. Their poor vascularity and large stromal component that has high fluid pressure leads to inefficient drug delivery to the pancreatic cancer cells.^{211,212} The TV was therefore segmented in this work to see if changes in perfusion as a result of nelfinavir therapy were greater in the tumour's core or periphery. The null hypothesis to be tested states that nelfinavir has no effect on tumour perfusion.

3.2 Methods

3.2.1 Patient selection

Imaging from patients enrolled into an ethically approved Phase II clinical study looking at the addition of the Akt inhibitor nelfinavir to CRT in patients with histologically confirmed LAPC (EUDRACT No: 2008-006302-42) was analysed. Inclusion criteria for the trial limited participation to those with locally advanced pancreatic adenocarcinoma or patients with resectable disease who were inoperable due to comorbidity with no evidence of MD.

FDG-PET imaging was available for the first 17 patients recruited in the study at the time of analysis. FMISO-PET and pCT were available for the last 6 patients recruited to the study.

3.2.2 Chemoradiotherapy schedule

The treatment schedule has been described previously¹⁸⁶ and is summarised in Figure 18(A). In short, gemcitabine 300 mg/m² and cisplatin 30 mg/m² were administered on the

Tuesday of the 1st, 2nd, 4th and 5th weeks of radiotherapy. In addition, nelfinavir was administered at a dose of 1250 mg twice daily. For the patients in the FDG-PET analysis group, nelfinavir was started 3 days before starting CRT until the last day of treatment. In the FMISO-PET/pCT group, nelfinavir was started 10 days before commencement of CRT to allow for sequential hypoxia and perfusion imaging before and during nelfinavir monotherapy. For each patient in the FDG-PET analysis, a 3D-conformal radiotherapy plan was made with a dose of 50.4 Gy in 28 fractions being delivered to the primary pancreatic tumour and elective regional lymph nodes, with a sequential boost of 9 Gy in five fractions to the GTV with a margin of 1 cm cranially, 2 cm caudally and 1.5 cm radially. A minimum of four 6–15 MV photon beams were used. For the 6 patients who had FMISO-PET/pCT imaging, a 5–7 field IMRT technique was used to deliver the same radiation doses to the volumes described above.

3.2.3 Planning CT

Following fasting for 2 h, the patient received 50 mL of water orally just prior to an exhale breath-hold CECT followed by a four-dimensional (4D) CT to inform target volume definition for radiotherapy planning. Patients were supine on a flat couch with knee rests, with arms above the head with a head support. The patient was scanned from above the dome of the diaphragm to the bottom of L4. A CT slice thickness of 2.5 mm was used.

3.2.4 FDG-PET/CT scanning

FDG-PET/CT was performed at baseline and 6 weeks after completing CRT. The FDG-PET imaging schedule is summarised in Figure 18(B). All scans were performed on a GE Discovery 690 (GE Healthcare, Buckinghamshire, UK). After fasting for 6 h and ensuring that the blood glucose was <10 mmol/L, FDG was injected at a dose of 4 MBq/kg (up to

600 MBq). PET acquisition was started after an uptake time of 90 min. Patients were scanned immobilised in the radiotherapy treatment position to aid accurate image coregistration. The whole body from below the eyes to the mid-femurs was scanned. Scans were performed in 3D with a scan time of 4 min at each bed position. For the CT phase, 120 kV automA (max 250 mA), noise index 25.0 0.5 s/rotation, pitch 0.984:1, 3.75 mm slice width was used. Attenuation-corrected PET images were used in the analysis.

3.2.5 FMISO-PET/CT scanning

The FMISO-PET imaging schedule can be seen in Figure 18(C). Nelfinavir 1250 mg orally twice daily was started 10 days prior to commencing CRT. All patients had FMISO-PET/CT and pCT 24–48 h prior to starting nelfinavir and again on day 6 or 7 of nelfinavir treatment.

FMISO-PET/CT and pCT were acquired using the same GE Discovery 690 scanner. Patients were cannulated in the dorsum of the hand to allow for blood sampling and in the antecubital fossa of the contralateral arm for tracer injection. They were scanned on a flat couch, immobilised with a personalised upper body vacuum bag with arms raised to aid later image registration between time-points. CT was performed to localise the pancreatic tumour (120 kV automA (max 250 mA), noise index 25.0 0.5 s/rotation, pitch 0.984:1, 3.75 mm slice width). A 370 MBq bolus of FMISO was injected 30 s after starting the PET acquisition. The dynamic PET images were acquired using variable time frames; 30 s prior to tracer injection followed by 12 x 5 s frames, six x 10 s frames, five x 30 s frames, 10 x 60 s frames and six x 5 min frames, resulting in a total acquisition time of 45 min. The patient then left the scanner and returned for a PET/CT 2 h and 4 h post injection. These late scans were performed with the patient in the same position, a CT was repeated followed by a 10 min PET acquisition. The CT scans were used for PET attenuation

correction and image coregistration. The attenuation-corrected images at 2 h and 4 h and the ordered subset expectation maximisation iterative reconstruction of the dynamic acquisition were used for analysis.

To allow for the calculation of a blood-derived arterial input function (AIF), blood was taken 4 min, 60 min, 2 h and 4 h post FMISO injection. The 4 min sample was venous blood. The remaining samples were considered to be arteriovenous samples as the hand was heated (in an in-house developed 'Hot Box' that increases the temperature of the hand using hot air) for 10 min prior to sampling to allow for increased venodilation and presumed reduction in arteriovenous transit time. Heating the hand to obtain an 'arteriovenous' blood sample is preferred as it avoids the need for arterial cannulation which is uncomfortable for patients and runs the, albeit small, risk of ischaemic damage to the hand and pseudoaneurysm formation. The hand-heating method has been demonstrated to give blood gas values in the arterial range,²¹³ suggesting that it can be used as a surrogate for arterial blood. Hand-heating is used routinely in metabolic studies as a safe alternative to arterial blood sampling as circulating metabolites in 'arteriovenous blood' following hand-heating are equal to those in arterial samples.^{214,215} The blood drawn from a dorsal hand vein after heating the hand was therefore used for tracer kinetic analysis.

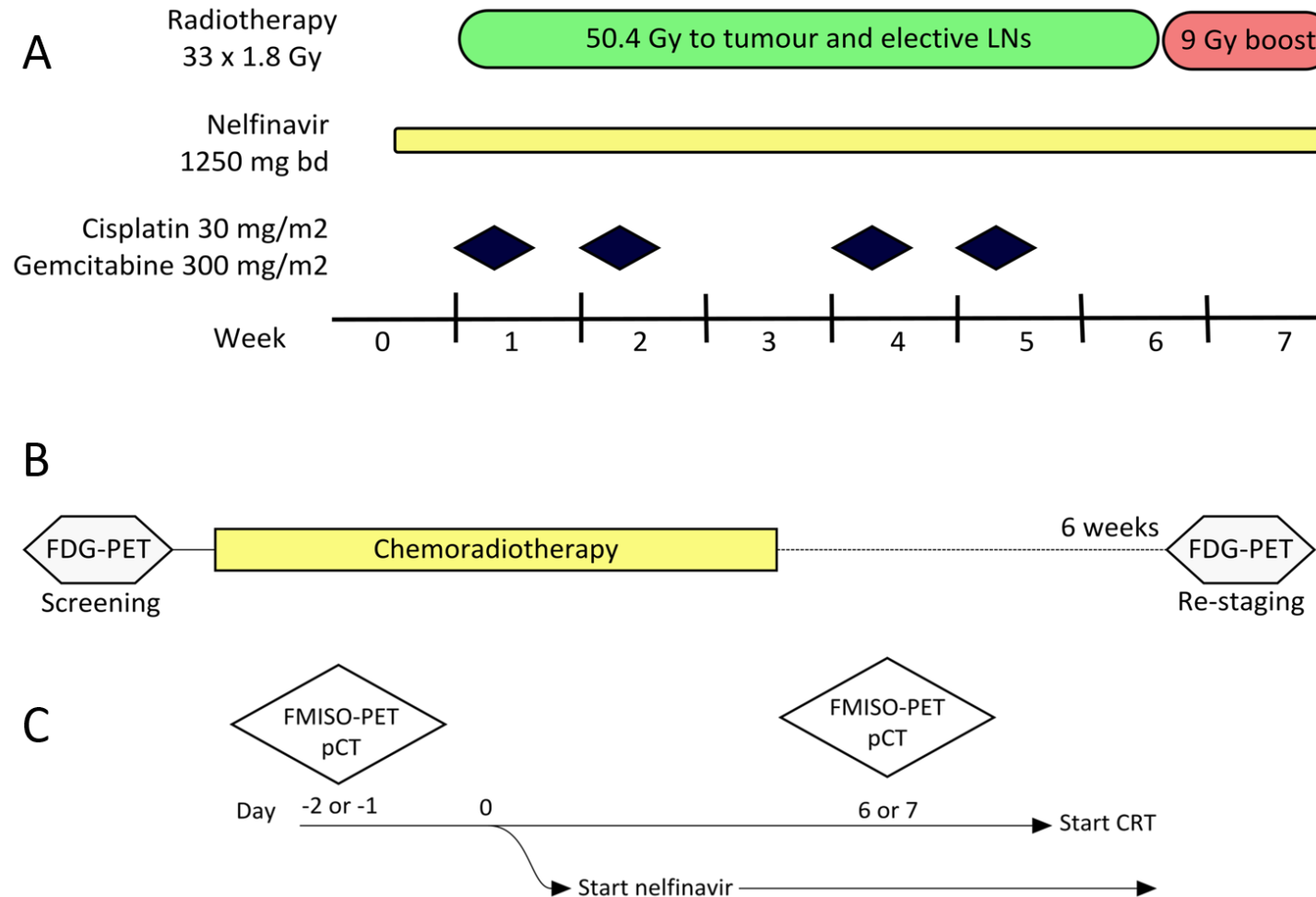


Figure 18: A summary of the ARC2 trial, including imaging substudies. The treatment interventions are summarised in (A). The timing of the FDG-PET/CT imaging in relation to the trial treatment can be seen in (B). (C) demonstrates the FMISO-PET and pCT imaging protocol which was carried out in the week prior to CRT (week 0 in Figure A). (LN = lymph nodes).

3.2.6 FDG-PET image analysis

3.2.6.1 Defining the tumour volume

The planning CT was loaded into Eclipse software (version 10.0.42, Varian Medical Systems, Palo Alto, CA, USA). The GTV was delineated by a radiation oncologist on the exhale breath-hold CECT. The FDG-PET images were not registered with the planning CECT during GTV delineation. All GTVs were reviewed and approved by a second radiation oncologist to reduce interobserver variability.

The CECT and FDG-PET images were imported into Mirada (Mirada Medical, Build 1.0.1.4, Oxford, UK) for non-rigid registration and the registered image imported back into Eclipse for overlap fraction calculation.

3.2.6.2 Thresholding based on standardised uptake values

SUVs were calculated using an elliptical ROI drawn around the area of increased uptake within the pancreas that excluded the liver and kidneys. The SUV_{max} within this ROI was recorded. Tumour segmentation was performed as a percentage of the SUV_{max} within the ROI. On the baseline FDG-PET this was performed at 40% and 50% of the SUV_{max} . On the post-treatment images 60%, 70%, 80% and 90% of the SUV_{max} were used. These values have been used previously.^{99,216} Because of a reduction in FDG uptake post CRT, segmenting the tumour using 40% or 50% of SUV_{max} generated volumes that were much larger than the residual TV. As the SUV_{max} had decreased, values below 60% of the SUV_{max} represented normal FDG activity rather than tumour retention. For these reasons, the tumour was segmented at >60% of the SUV_{max} post CRT.

3.2.6.3 Image coregistration

The Discovery 690 PET and CT images share the same intrinsic frame of reference, so the CT component was used for registration with the planning CT scan. An automatic rigid registration, followed by a non-rigid registration, was performed using Mirada software. Bony anatomy was visually checked to ensure that vertebrae were not misaligned and the quality of the entire registration was verified for gross errors. The deformation field from the verified CT-CT registration was then applied to the PET data and contours were propagated from the PET to the planning CECT.

3.2.6.4 Assessment of spatial relationship of the SUV-derived subvolumes

The percentage of intersection between the volumes derived from the baseline 40% and 50% of SUV_{max} (Pre40% and Pre50% respectively) with the clinician-defined GTV were assessed by quantifying the overlap between the two volumes and expressing that as a percentage of the GTV. The percentage intersection of the post-CRT volumes with the pre-CRT volumes was calculated and expressed as a percentage of the smaller post-CRT volume. This method of defining an overlap fraction has been previously reported⁹⁹ and is summarised in Figure 19.

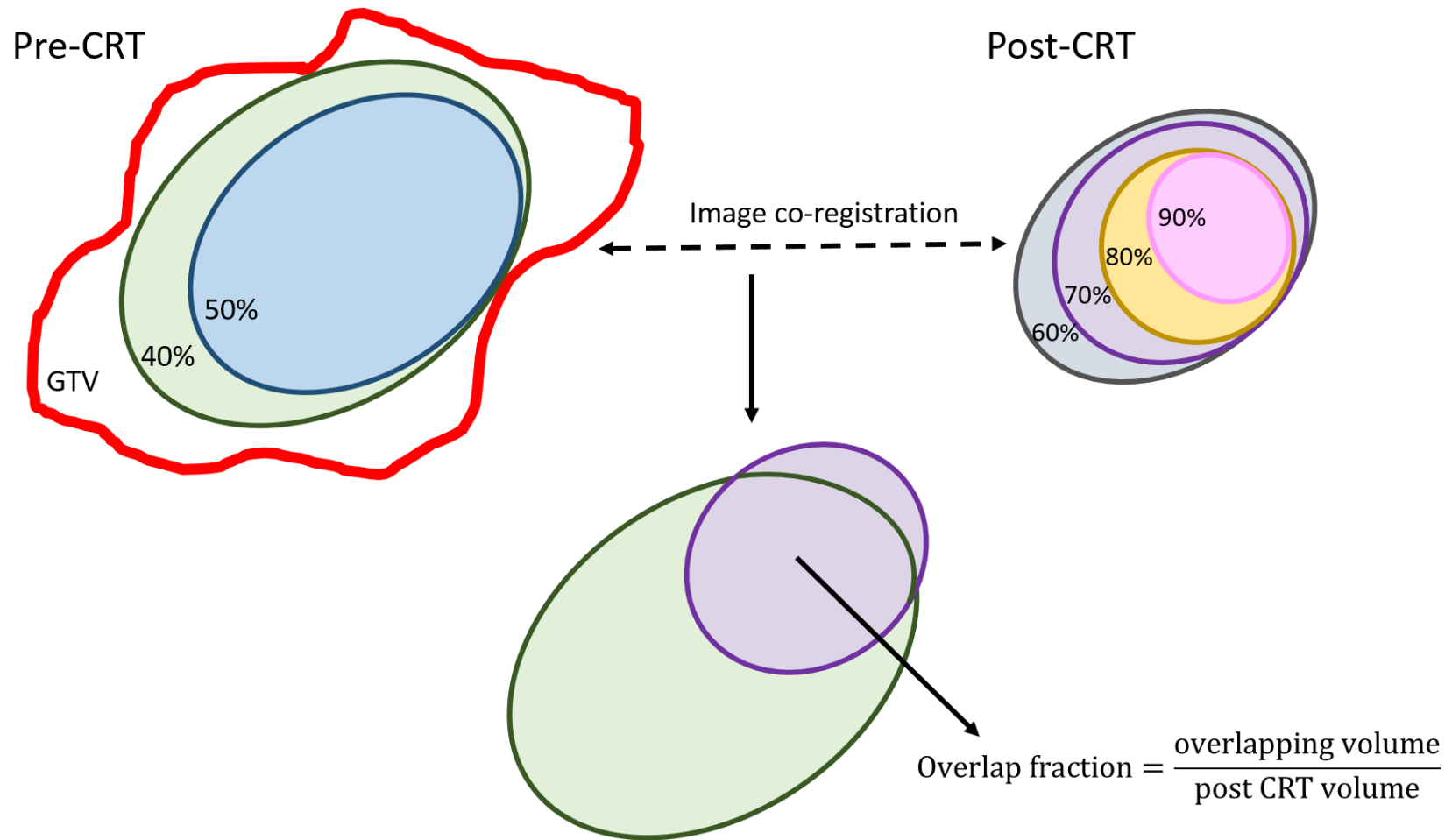


Figure 19: A representation of how the overlap fraction of pre- and post-CRT FDG uptake was calculated. Within the pre-CRT GTV, volumes delineated by 40% and 50% of SUV_{max} were generated. Post-CRT volumes defined by 60%, 70%, 80% and 90% of SUV_{max} were also generated. Following image registration, the volume of overlap between the pre- and post-CRT FDG-PET-derived volume was obtained and the overlap fraction was calculated as shown.

3.2.7 FMISO-PET image analysis

3.2.7.1 Region of interest definition

The attenuation-corrected, static acquisition PET/CT acquired 4 h post tracer injection (4 h FMISO-PET) was used to characterise maximum and peak FMISO uptake values. The CT component of the PET was rigidly registered to the CECT used for radiotherapy planning in Eclipse (version 13, Varian Medical Systems, Palo Alto, CA, USA). The GTV delineated on the planning CT was propagated to the CT component of the PET to generate a ROI labelled as 'tumour'. The paraspinal erector muscles and aortic lumen (a 2 mm contraction of the aortic contour as outlined on the CECT) were outlined on all tumour-containing slices of the CT and were labelled 'muscle' and 'blood' respectively. The 4 h PET/CT from the second imaging time-point (day 6 or 7) was rigidly registered to the first PET/CT. Unmodified ROIs were then propagated to the second PET/CT and checked for accuracy.

To allow for a pharmacokinetic analysis, the CT component of the 4 h PET was rigidly registered with the CT component of the dynamic and 2 h PET and the ROIs were copied across. Soft tissue matching at the site of the tumour was visually assessed and prioritised for each registration to maximise accurate tumour matching.

3.2.7.2 FMISO uptake quantification

The 4 h PET and the ROIs were imported to PMOD software (PMOD Technologies, Zurich, Switzerland).¹⁹⁸ The maximum and peak (mean activity within the 1 cm³ sphere with the highest activity) SUV (SUV_{max} and SUV_{peak} respectively), maximum and peak T:M (T:M_{max} and T:M_{peak} respectively) and maximum and peak T:B (T:B_{max} and T:B_{peak} respectively) were calculated at each time-point.

To define subvolume of the tumour that could be described as 'hypoxic', the volume of tumour with an uptake value greater than reference tissue was used. Two previously recognised thresholds of FMISO uptake that are thought to represent hypoxia were used: a T:M > 1.3^{95,170-172} and a T:B > 1.4.^{153,177,178,183} The volume of tumour with FMISO uptake greater than these thresholds was calculated on both the pre- and post-nelfinavir imaging. Segmentation of the tumour and the required segmentation of the normal reference tissues (muscle and blood) can be seen in Figure 20.

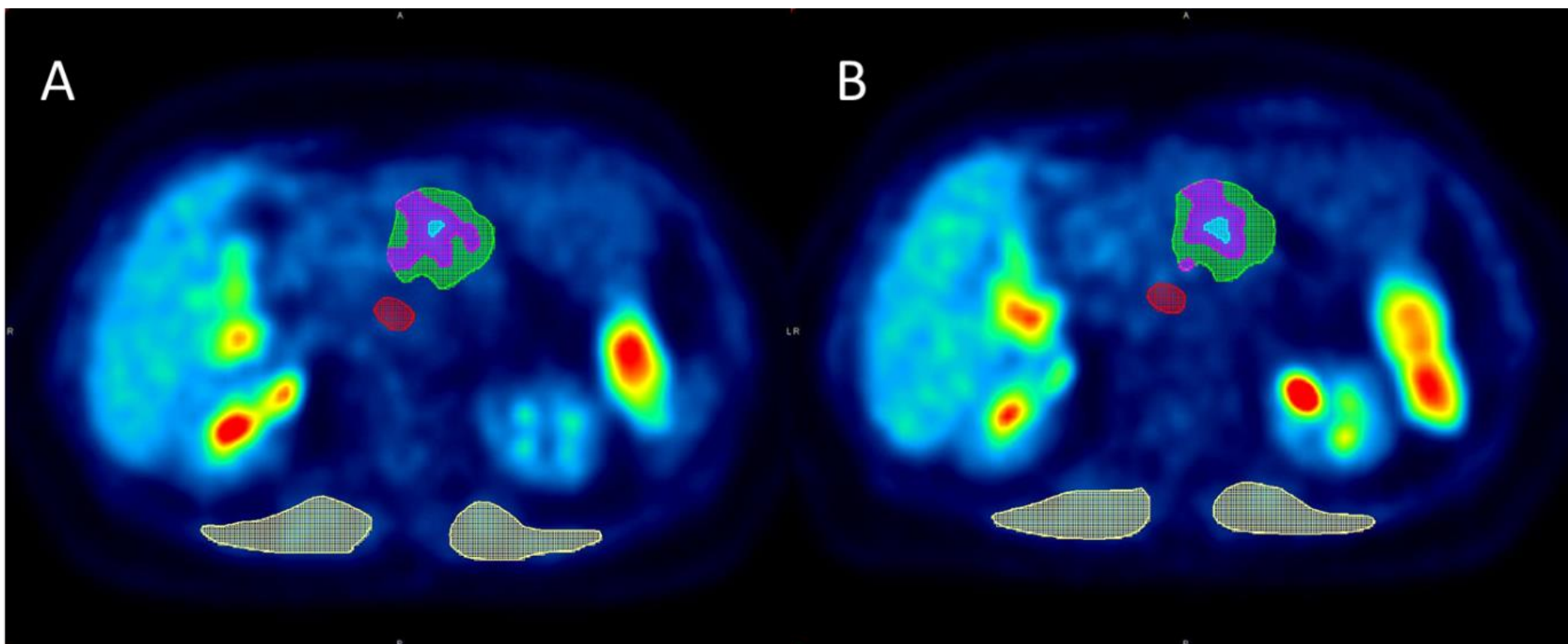


Figure 20: Transaxial sections from FMISO-PET images from Patient C, both pre- (A) and post (B) nelfinavir. The tumour ROI (green) was used to quantify uptake, above that seen in blood (red) and muscle (white). The uptake volumes $T:M > 1.3$ (pink) and $T:B > 1.4$ (light blue) are demonstrated.

The SUV_{peak} sphere was saved as a ROI, and the coordinates of its central voxel in relation to the coordinates of the centre of mass of the tumour ROI were recorded. As the tumour ROI was volumetrically identical at each time-point, the pre-nelfinavir TV could be matched to the post-nelfinavir TV by normalising the coordinates of the centre of mass on the second scan to the first. MatLab (version 2013a, MathWorks, Natick, MA, USA) was then used to calculate the distance between the centres of the SUV_{peak} at the two time-points. This distance is demonstrated in Figure 21.

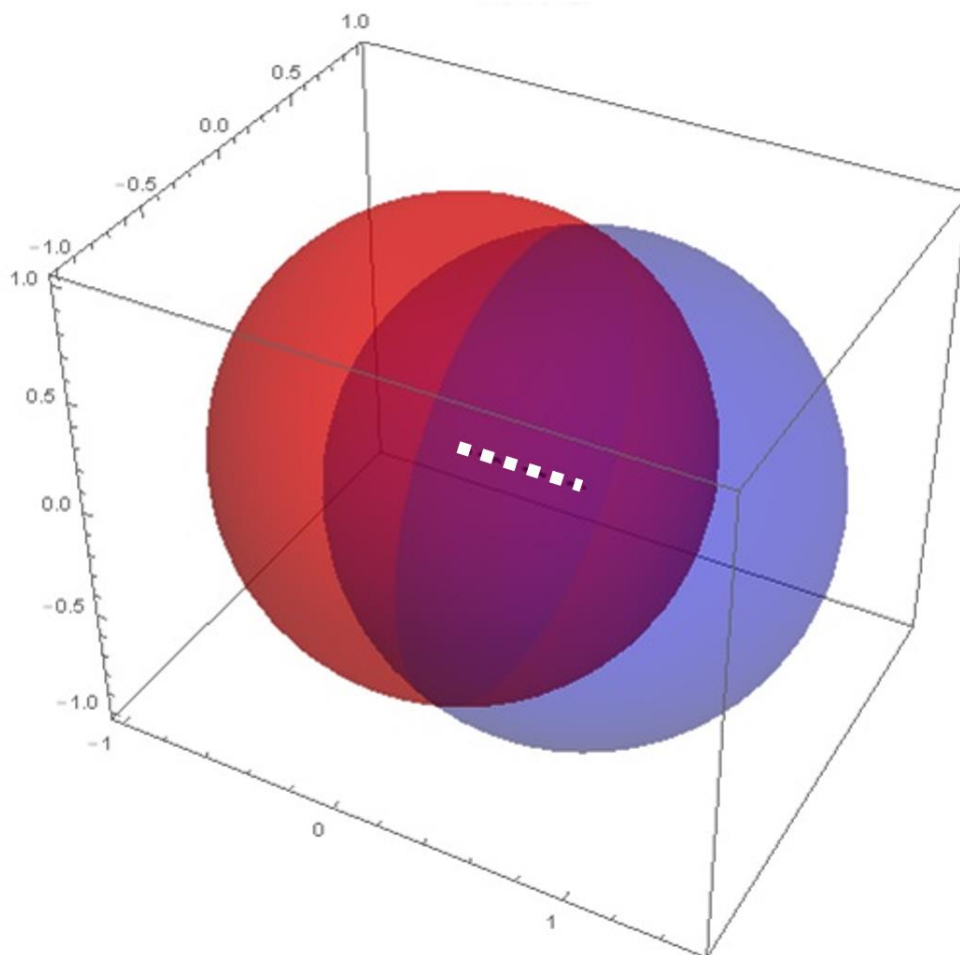


Figure 21: The 1 cm^3 spheres with the highest mean FMISO activity (SUV_{peak}) on the pre-nelfinavir FMISO-PET (red) and post-nelfinavir FMISO-PET (blue) were plotted on the same axis after matching the tumour ROIs. The distance between the centre points of the SUV_{peak} volumes between time-points was calculated (white dotted line).

3.2.7.3 Pharmacokinetic analysis

The dynamic, 2 h and 4 h PETs with their associated ROIs were loaded into PMOD. TACs were generated for the tumour and blood. Measured activity within the tumour ROI over the first 45 min of scanning plus that measured at 2 h and 4 h generated 4 h FMISO TACs. The TAC data were then exported as a text file and imported into MatLab. The blood TAC was then used to generate an AIF using a technique that has previously been described.²¹⁷ This method corrects for partial volume effect and incorporates decay-corrected measured blood tracer activity to image-derived measured activity before fitting the data to a one-compartmental model.

FMISO transport kinetics was then quantified by fitting the AIF information and tumour TACs to two-, three- and four-compartment models. An illustration of the parameters that can be derived from two- and three-compartmental models and how they describe FMISO uptake and return to the circulation can be seen in Figure 22. In the three-compartment model, parameters K_1 and k_2 describe movement of FMISO to and from the first tissue compartment from the circulation. Movement from this first tissue compartment to a second tissue compartment (in which FMISO can be considered to have been retained) is described by k_3 and k_4 . The measured TAC was fitted to model data in a way that minimised the difference between the model and TAC data using departmental software (developed by Dr Sara Hackett using MatLab based on the method and model governing equations derived by Bruehlmeier *et al.*¹⁶³). Once the data had been fitted to the model, it was possible to quantify the kinetic parameter values. The following models were fitted to the data: two-compartment (C), four-parameter (K) (2C4K), 2C3K, 3C6K, 3C5K, 4C8K, 4C7K. Some of these models, for example the 3C5K model, eliminate the potential for the return

of reduced FMISO to return to the circulation by setting the relevant parameter (e.g. for the 2C3K model, k_4) to zero.

Once the model that best describes the tumour TAC was found, the parameter that describes the retention of FMISO in a hypoxic cell was quantified in each of the images (e.g. in a two-compartment model, k_3 is of interest in a three-compartment model, it would be k_5).

To assess how closely this analysis of the dynamic imaging compares with the more standard method of assessing hypoxia using the 4 h FMISO-PET, a correlation was sought between the dynamic parameter associated with FMISO intracellular retention and maximum and peak values seen at 4 h post tracer injection.

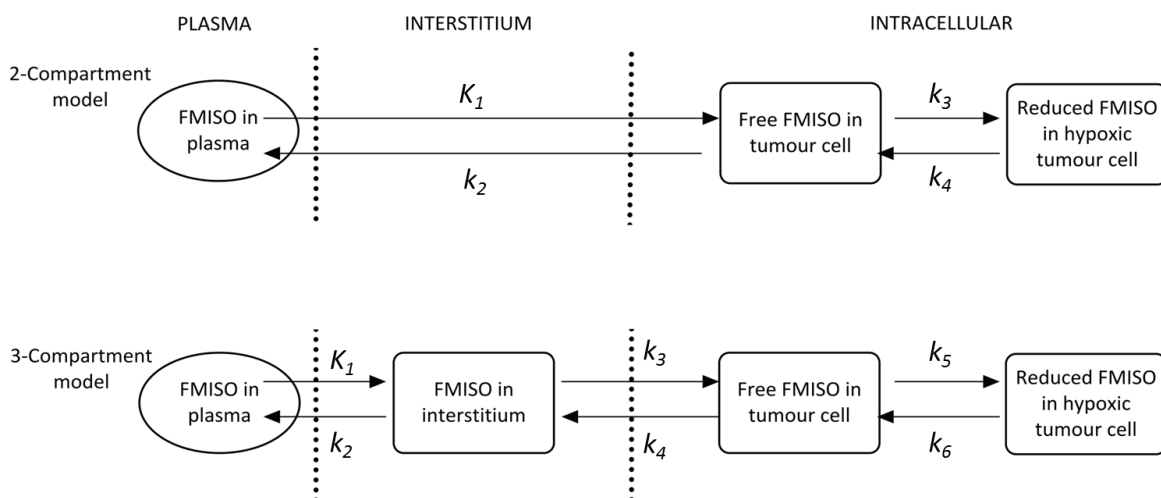


Figure 22: Two- and three-compartmental models describing movement of FMISO to and from the circulation into the tumour.

The first step in choosing which compartmental model to use to describe the observed tumour TACs is to assess the goodness of fit of the model to the TAC using the runs test. The runs test generates a 'pass' or 'fail' outcome based on how well the model fits the measured data. Measured data points that exceed the model value are assigned '+', while

measured values that are lower than the model value are assigned ‘-’. Prolonged series of ‘+’ or ‘-’ suggest that the difference between the model and the measured data is not random, suggesting a poor model fit.²¹⁸ As the runs test does not take into account the magnitude of the difference between the measured data and the pharmacokinetic model, additional testing is required before deciding which model most accurately represents the data. The fit of each model to the image-derived TAC was then compared with that of other models using Akaike information criteria (AIC) and Bayesian information criteria (BIC); in general, the model with the lowest AIC/BIC is the most likely to predict new data.²¹⁹ This information from all 12 FMISO-PET TACs was used to decide which model to use to derive parameter values. Two-, three- and four-tissue compartment models were fitted to the tumour TAC. The simplest model with the lowest AIC/BIC was selected. A model with a large number of parameters and compartments will, undoubtedly, have the lowest AIC/BIC values. An understanding of the physiology of FMISO diffusion and retention (as demonstrated in Figure 8 in Chapter 1) was also taken into account. Parameter values were therefore derived from the simplest model that was able to describe the data, but not so detailed that it was over-fitting to noise in the observed TAC data.

3.2.7.4 Characterising hypoxia with dynamic FMISO-PET

Once a model that adequately described tumour FMISO kinetics had been found, FMISO retention within the tumour ROI could be quantified. The percentage change in the FMISO retention parameter was calculated. This change could then be used to see how changes in FMISO retention (and inferred hypoxia) relate to changes in perfusion as characterised by pCT.

3.2.8 Perfusion CT analysis

3.2.8.1 Image acquisition

Following straight on from the 4 h late acquisition PET image, a pCT was performed with the patient in the same, immobilised, position. An attempt at breath-holding was made, but it was acknowledged that breath-holding for the whole scan acquisition time (45 s) would not be possible for the majority of patients. A breath was therefore encouraged at 25 s with the patient returning to exhale breath hold for the remainder of the scan time.

A pre-contrast CT was taken (120 kV, automA [max 600 mA], noise index 32.0 0.5 s/rotation, pitch 0.984:1, 6.25 mm slice width). The pancreatic tumour was localised and a 40 mm (craniocaudal) scanning area for the perfusion sequence was identified; 70 mL of contrast (Omnipaque 300, GE Healthcare) was injected at a rate of 5 mL/s followed by a 25 mL saline flush, which was injected at the same rate. A continuous acquisition with a 1.0 s rotation time and 8 images/rotation was obtained for a 45 s exposure (120 kV, 60 mA, noise index 7.8, 5.0 mm slice thickness).

3.2.8.2 Motion correction

Dr Bartlomiej Papiez performed motion correction to the pCT images using an intensity-based deformable image registration method based on a non-parametric image deformation (Diffeomorphic Demons) modified by use of the normalised gradient fields²²⁰ using software developed in house. The normalised gradient fields is the contrast-invariant representation of volume, which is used during estimation of the deformation vector field to handle intensity changes caused by contrast uptake. Additionally, the deformable image registration takes advantage of the multiresolution framework with three levels with the final spacing equal to the original volume spacing. The maximum number of iterations for

each level is fixed to 25, and at each resolution level the standard deviation of the Gaussian smoothing was 2.8, 1.4 and 0.7 mm. This method has previously been applied to reduce the impact of motion on the analysis of lung imaging.²²¹

3.2.8.3 Pharmacokinetic analysis of pCT data

Perfusion parameter maps were generated using the GE Advanced Workstation 4.0 using the pancreatic perfusion protocol. Non-motion-corrected and motion-corrected images were analysed. An AIF was generated by placing a ROI within the aorta at the level of the pancreatic tumour. The resultant AIF curve was visually assessed and the timing of the last pre-enhancement image was verified before generating perfusion-related parameter maps. An example of the AIF for Patient 1 can be seen in Figure 23. Parameter maps for blood flow (BF) and blood volume (BV) were then generated. A smoothing filter with a kernel width of 3.5 mm was automatically applied to the image to reduce noise.

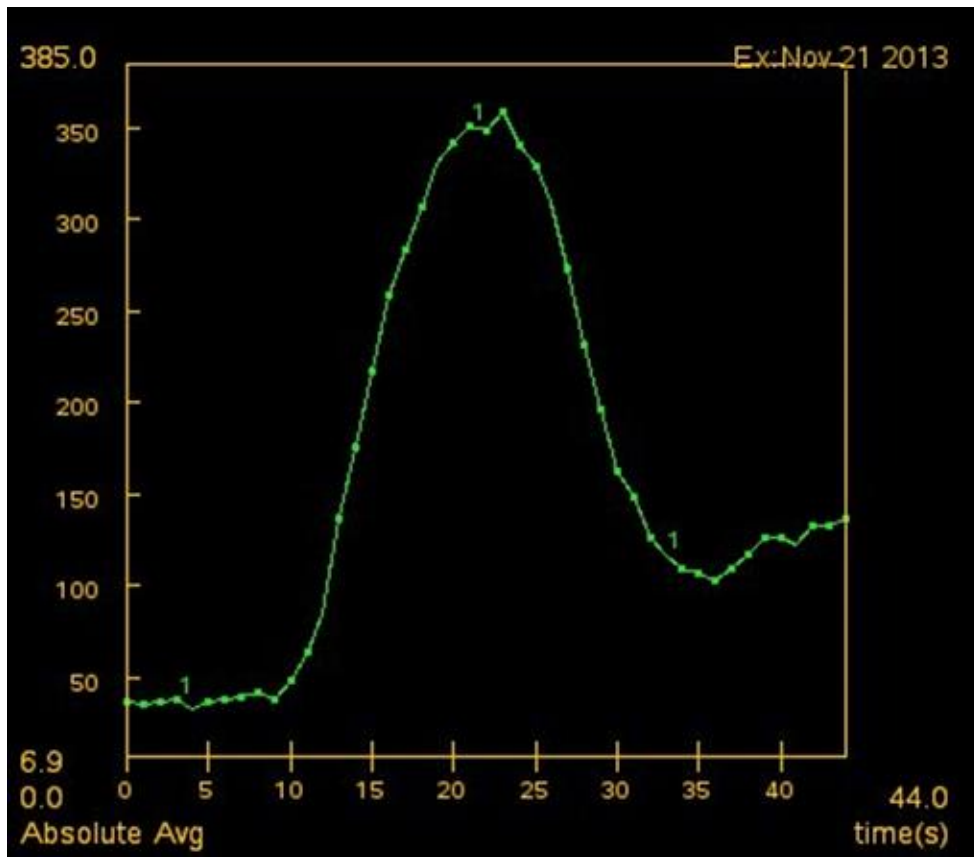


Figure 23: An example of a pCT, image-derived arterial input function. A ROI within the aortic lumen at the level of the pancreatic tumour was used to generate the contrast time activity curve.

The necessary steps required to generate ROIs labelled ‘tumour’, ‘core’ and ‘periphery’ are outlined in Figures 24 and 25. The tumour was outlined on the contrast-enhanced planning CT in Eclipse. The pre-contrast component of the pCT was then rigidly registered to the contrast-enhanced planning CT using Mirada. The tumour ROI was then propagated on to the pre-contrast CT. The pre-contrast CT shares the same frame of reference as the BF parameter map. The tumour ROI volume could then be transferred to the parameter maps. Extreme values were then excluded from the volume to exclude non-perfused non-tumour tissue and large blood vessels contained in ROI (see Figure 25 D). A new ‘tumour’ ROI was then used for pCT parameter quantification. The tumour ROI was contracted by 3 mm in all directions to obtain a ROI called ‘core’. This was then subtracted from the tumour ROI

to generate a ROI called 'periphery'. The decision to use a 3 mm contraction to define the tumour periphery was taken in light of the spatial resolution of the CT images and as a first step in detecting heterogeneity in the response of tumour perfusion as a result of inadequate drug entry to the core of the tumour compared with the more 'superficial' components of the tumour. As all parameter maps share the same frame of reference, the ROIs were transferred on to the blood volume parameter maps for further quantification. This process was performed on both unprocessed pCT data and motion-corrected images.

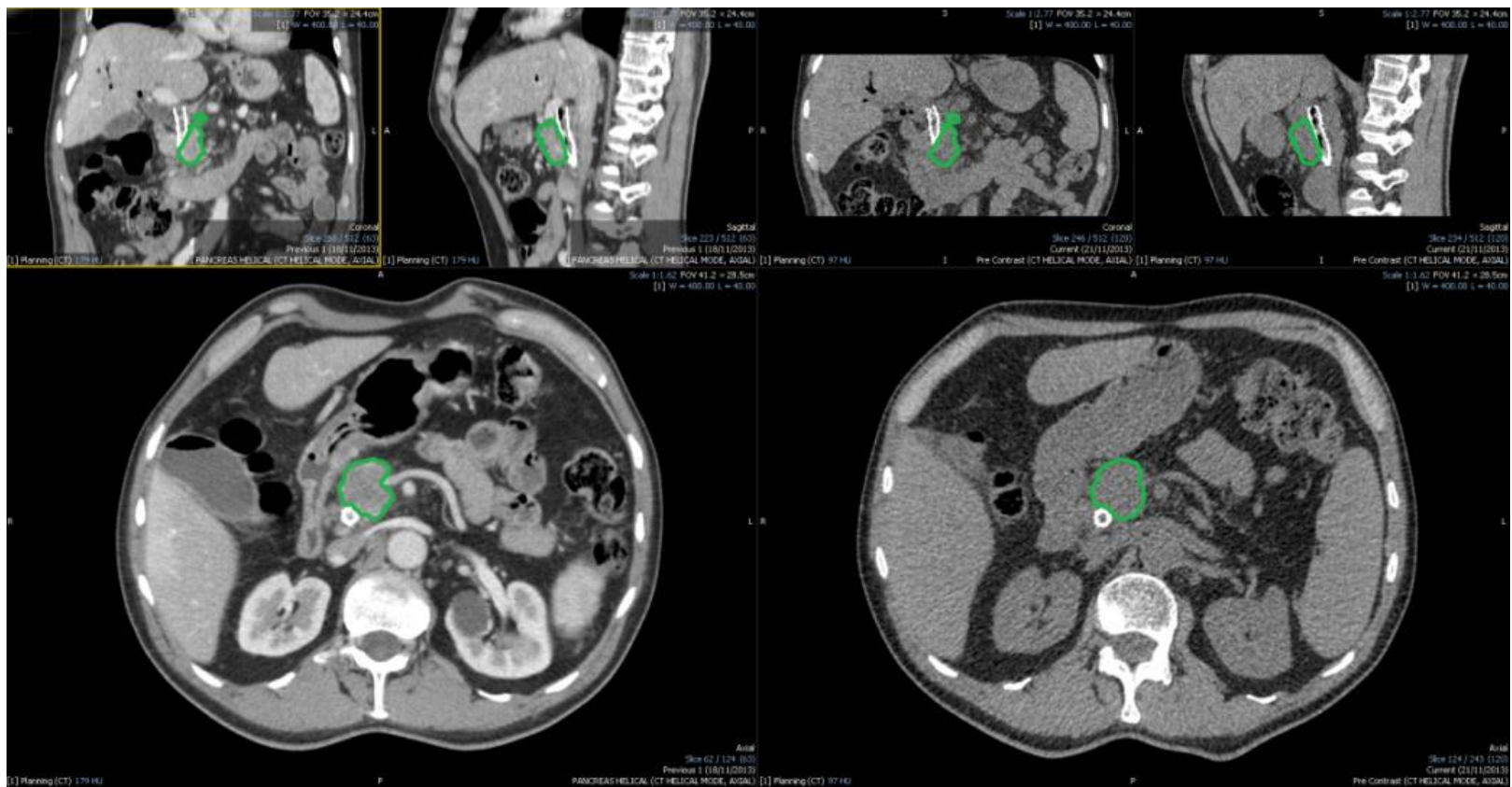


Figure 24: Rigid registration of the CT component of the FMISO-PET/CT with the contrast-enhanced planning CT in Mirada. If necessary, the tumour ROI (green) could be modified on the non-enhanced CT if it was seen to extend beyond the tumour margin.

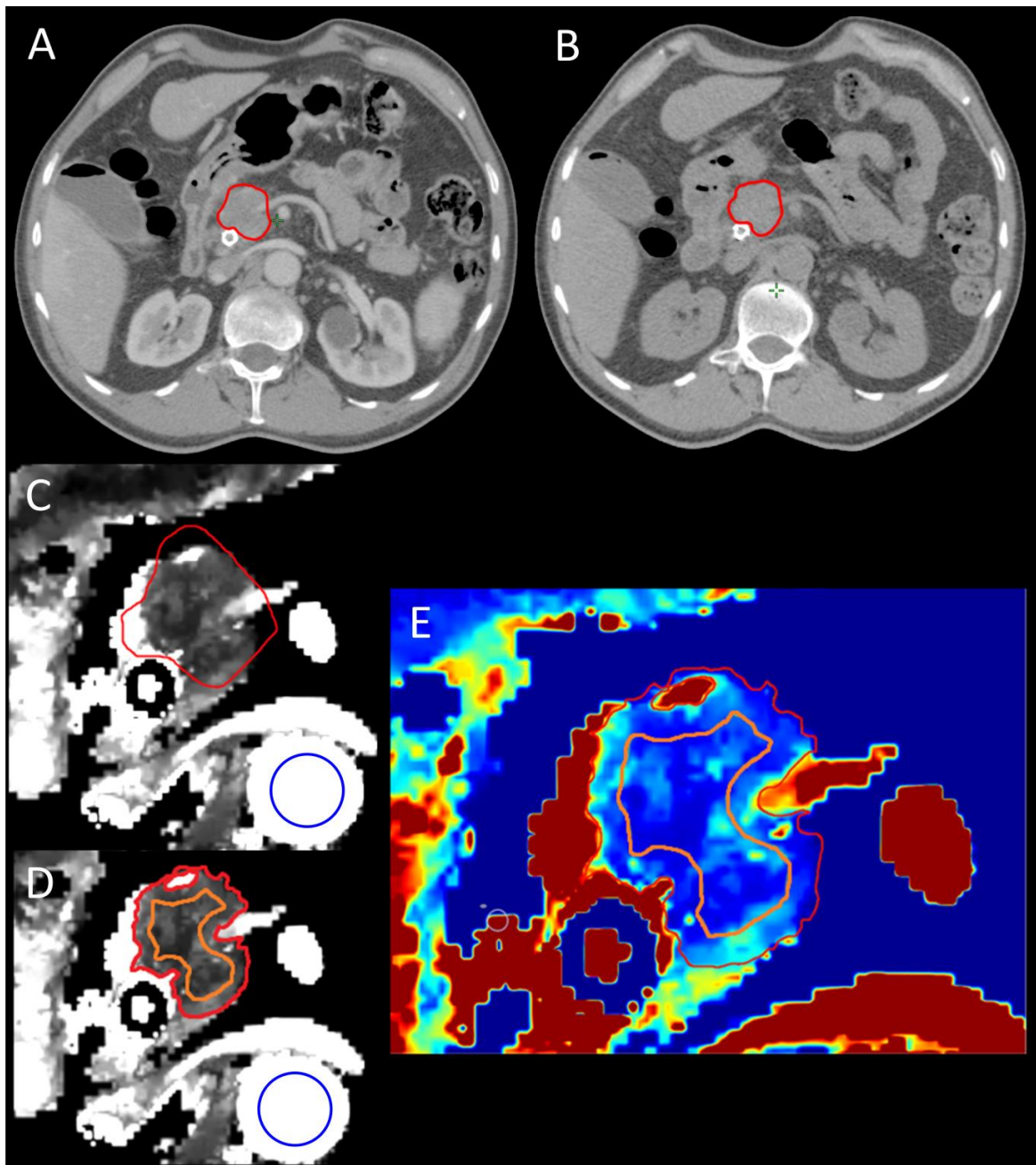


Figure 25: (A) Axial section from the contrast-enhanced planning CT on which the tumour ROI was outlined (red). (B) The pre-contrast CT from the pCT was registered to the planning CT and the tumour ROI was copied across. (C) A blood flow parameter map for Patient 2. The aorta ROI (blue) used for generating an AIF can be seen. (D) Modification of the tumour ROI for perfusion parameter quantification. The GTV was edited to remove extreme values that were likely to represent non-viable, non-tumour tissue and blood vessels to generate the final tumour ROI. This was contracted by 3 mm in all directions to produce a ROI labelled 'core' (orange). The tumour ROI, excluding core was labelled 'periphery'. (E) The resultant volumes were used to quantify the perfusion parameters.

3.2.9 Statistical analysis

Statistical analysis of the FDG-PET SUV_{max} pre- and post CRT was performed by running a Mann Whitney U test in GraphPad Prism 5 (version 5.03, GraphPad Software Inc., La Jolla, CA, USA). Pre- and post-nelfinavir FMISO-PET parameters and BF and BV parameters when derived from unprocessed and motion-corrected pCT were compared using the Wilcoxon matched-pairs signed-rank test using SPSS Statistics 22 (IBM, Portsmouth, UK). The correlation of the k_3 parameter derived from pharmacokinetic modelling using a three-compartmental model with the 4 h FMISO-PET-derived parameters at baseline and post CRT was characterised by Spearman's rho correlation coefficient and calculation of the p value of the correlation using SPSS.

3.3 Results

3.3.1 Results of the FDG-PET/CT analysis

3.3.1.1 Patient details

Of the 17 patients included in the FDG-PET analysis, 4 had a complete metabolic response (cMR) at 6 weeks following CRT, one patient's tumour was not FDG avid, one patient did not have a post-CRT FDG-PET, one patient had diffuse FDG uptake that could not be differentiated from surrounding normal tissue, one patient progressed with new liver metastases on the post-CRT FDG-PET and one patient had a serum glucose >20 mmol/L at both scanning time-points, making image interpretation impossible. This left nine pairs of scans that could be included in the analysis of the spatial distribution of pre- and post-CRT FDG uptake. Sixteen patients received CRT as planned. One patient's treatment was discontinued after he had received 45 Gy to the GTV and elective lymph nodes because of a complication unrelated to this treatment.

3.3.1.2 Assessment of tumour response

There was a significant decrease in SUV_{max} following CRT (pre-CRT median SUV_{max} 8, range 2.6–15.6, post-CRT median 3.6, range 1.6–7.9; $p = 0.001$, Figure 26). Pre- and post-treatment SUV_{max} values for all patients are shown in Table 7.

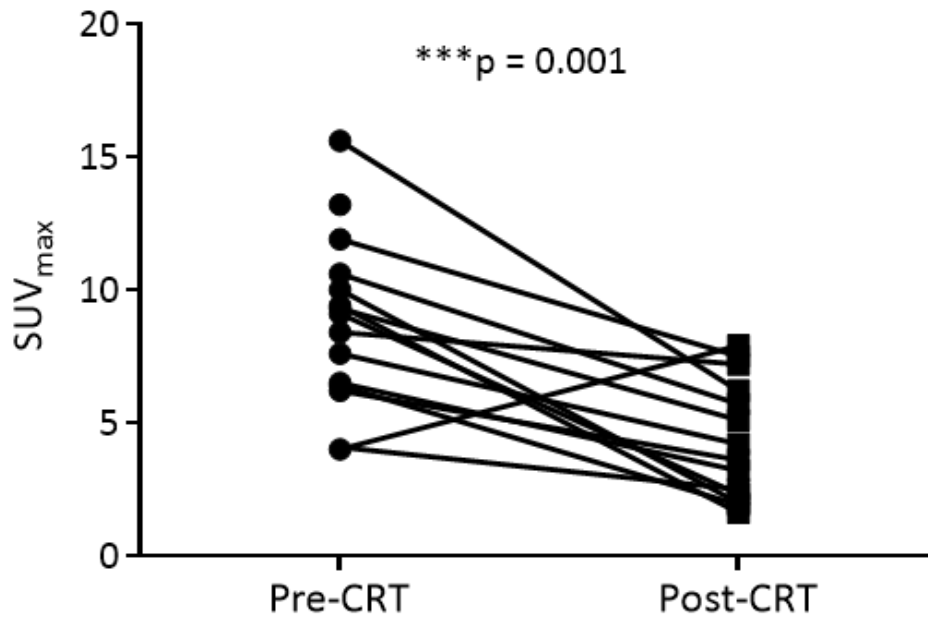


Figure 26: Pre- and post-CRT SUV_{max} values.

Table 7: Pre- and post-treatment SUV_{max} values and absolute volumes of the GTV and volumes derived from segmentation of the pre- and post-treatment FDG-PET/CTs.

Patient	Pre-treatment	Post-treatment	Absolute volumes (cm ³)						
	SUV _{max}	SUV _{max}	GTV	Pre40%	Pre50%	Post90%	Post80%	Post70%	Post60%
1	7.6	4.2	57.6	28.9	19.8	0.2	1.6	6.9	14.7
2	8.4	7.2	80.2	31.6	18.7	0.1	0.3	1.7	16.7
3	6.4	mCR	14.5	7.4	3.8				
4	10.6	5.7	41.9	15.6	9.0	0.1	0.6	2.3	4.9
5	15.6	6.2	44.9	15.7	10.5	0.1	0.1	0.9	2.6
6	13.2	±	55.2	8.1	3.4				
7	6.2	3.6	38.0	22.4	12.1	0.1	0.3	1.6	4.4
8	11.9	7.5	27.2	13.4	5.4	0.1	0.6	1.4	2.6
9	4.0	7.9	28.6	§	§	§	§	§	§
10	9.1	mCR	49.9	34.2	9.2				
11	10.0	mCR	19.3	4.8	2.5				
12	9.4	mCR	14.0	6	3.2				
13	¢	¢	10.6	¢	¢	¢	¢	¢	¢
14	4.1	2.5	22.3	11.0	5.9	0.1	0.3	2.2	4.0
15	¤	¤	40.8	¤	¤	¤	¤	¤	¤
16	9.3	5.1	32.9	21.8	15.7	0.2	1.3	4	7.6
17	6.5	3.2	26.6	19.0	11.8	0.2	1.6	5.5	11.4
Median	8.0***	3.6*** p = 0.001							
Mean^			41.3	19.9	12.1	0.1	0.7	2.9	7.7

mCR Metabolic complete response. ¢ Not FDG avid. § Diffuse FDG uptake making delineation of a ROI impossible. ¤ Elevated serum glucose, scans therefore could not be interpreted. ± Not done. *Difference in pre- and post-treatment SUV_{max} p = 0.0006. ^ Only values included in spatial correlation analysis included.

3.3.1.3 Relationship of the pre-CRT FDG-PET-derived subvolumes and the clinician-defined GTV

The mean GTV volume was 41.3 cm³ (range 22.3–80.2 cm³), mean Pre40% and Pre50% volumes were 19.9 cm³ and 12.1 cm³ respectively. The intersection between the Pre40% volume and the GTV was a mean of 50.8% (range 35.0–59.0%) of the GTV, while the Pre50% volume was a mean of 30.3% (range 19.9–34.4%) of the GTV (see Figure 27).

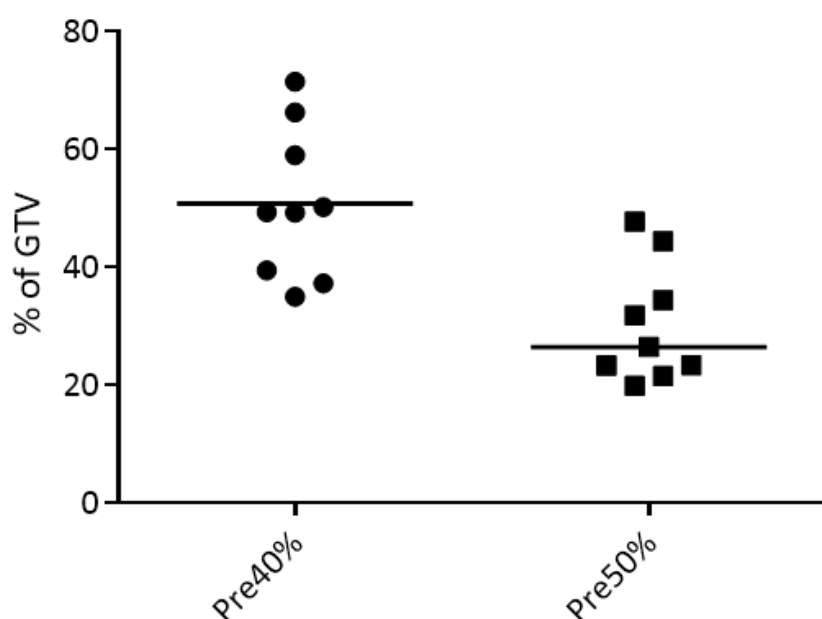


Figure 27: Pre-treatment volumes defined by 40% of SUV_{max} (Pre40%) and 50% of SUV_{max} (Pre50%) expressed as proportion of the GTV (line at mean).

3.3.1.4 Degree of agreement between the post-CRT areas of FDG uptake and the pre-CRT SUV40% and SUV50%

Figure 28 demonstrates how the volumes derived from the pre-CRT FDG-PET relate to the post-CRT volumes and to the GTV on the planning CECT. Patient 1 demonstrates the effect of tumour shrinkage on the relationship between the pre- and post-CRT volumes. While most of the residual metabolic activity is contained in the Pre40% volume, tumour

shrinkage meant that the tumour appeared to have moved in a caudal direction following CRT. Patient 4 shows an excellent correlation between pre- and post-CRT metabolic activity with all FDG-avid areas being contained within the Pre40% volume and the GTV. Patient 8 also shows an excellent agreement between the pre- and post-CRT metabolic activity, but the Pre40% volume covers a large proportion of the GTV (49.3%). Figure 29 demonstrates the percentage of the residual FDG uptake volumes contained within the Pre40% and Pre50% volumes averaged over all the patients. The absolute values can be found in Table 8.

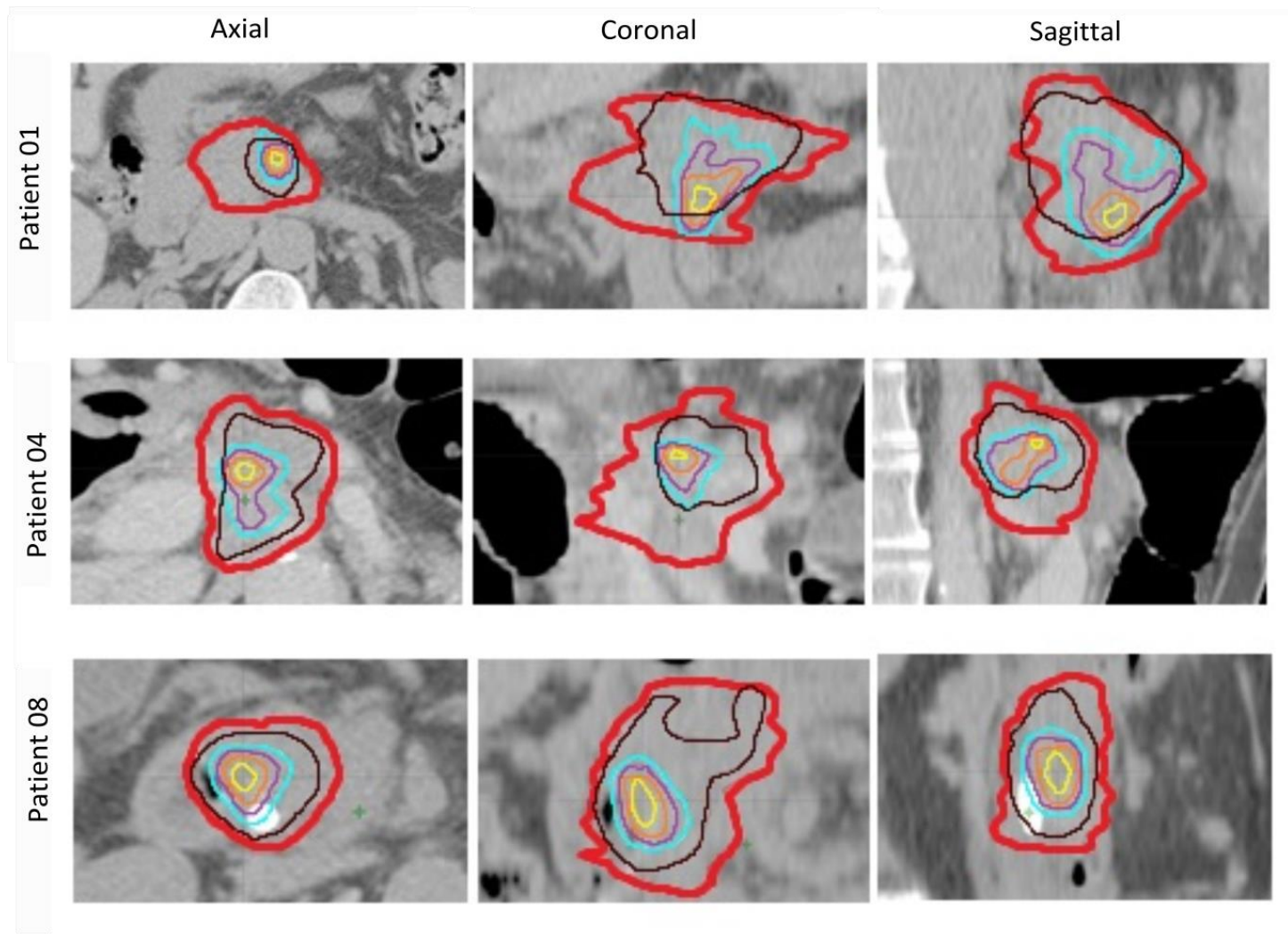


Figure 28: Relation of the GTV (red) and Pre40% (brown) to the Post60% (cyan), Post70% (purple), Post80% (orange), Post90% (yellow) in three representative patients.

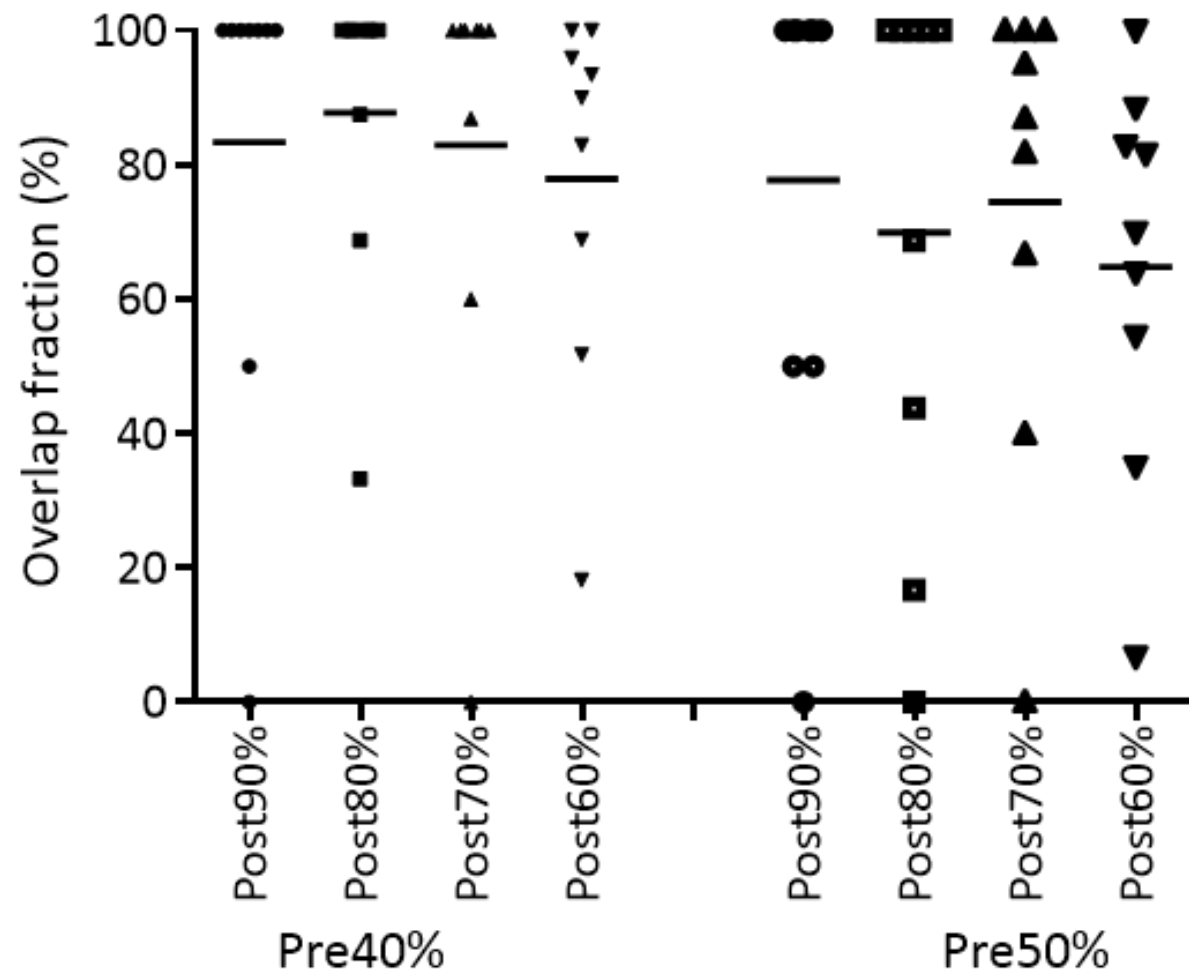


Figure 29: Degree of overlap between the Pre40% and Pre50% subvolumes and the post-CRT subvolumes. Lines represent the mean % intersection of the post-treatment volume with the volume delineated by 40% or 50% of SUV_{max} pre-CRT. Individual data points are shown to demonstrate the range of values.

Table 8: Degree of overlap between the post-treatment FDG-PET-derived volumes and the pre-treatment 40% and 50% of SUV_{max} volumes.

	% intersection of the following volumes							
	Pre-treatment 40% of SUV _{max} with:				Pre-treatment 50% of SUV _{max} with:			
Patient	Post90%	Post80%	Post70%	Post60%	Post90%	Post80%	Post70%	Post60%
1	100.0	87.5	87.0	83.0	50.0	68.8	66.7	64.0
2	100.0	100.0	100.0	68.9	100.0	100.0	100.0	54.5
4	100.0	100.0	100.0	95.9	100.0	16.7	87.0	81.6
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	88.5
7	0.0	0.0	6.3	18.2	0.0	0.0	0.0	6.8
8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
14	100.0	100.0	100.0	90.0	100.0	100.0	81.8	70.0
16	100.0	100.0	100.0	93.4	100.0	100.0	95.0	82.9
17	50.0	68.8	60.0	51.8	50.0	43.8	40.0	35.1
Mean	83.3	84.0	83.7	77.9	77.8	69.9	74.5	64.8

3.3.2 Results of the FMISO-PET/CT analysis

3.3.2.1 Patient details and concordance with the imaging protocol

Patient demographics and tumour characteristics of the 6 patients who had FMISO-PET and pCT imaging can be seen in Table 9. These patients were recruited to the study later than the 17 patients whose FDG-PET imaging has already been discussed.

Median age was 66 years (range 56–68) and 50% of the patients were male. Median tumour volume was 22.3 cm³ (range 11.3–34.9 cm³). All patients had T4 disease because of blood vessel involvement. Four of the tumours were in the pancreatic head, and one each in the uncinate and pancreatic neck. Two patients had peripancreatic lymph node

involvement, but hypoxia and perfusion was not assessed in the lymph nodes in this analysis.

Table 9: Patient characteristics for the hypoxia and perfusion imaging study

Patient	Age	Sex	Tumour location in pancreas	TNM stage	Tumour maximum diameter (cm)	Tumour volume (cm ³)
A	65	M	Neck	T4 N0 M0	4.3	20.8
B	65	M	Head	T4 N0 M0	4.7	23.5
C	68	F	Body	T4 N0 M0	4.9	34.9
D	56	F	Head	T4 N1 M0	3.2	11.3
E	67	M	Uncinate	T4 N0 M0	3.4	13.5
F	68	F	Head	T4 N1 M0	4.5	34.0

All patients had imaging as outlined in Figure 18(C) in the week before starting CRT. Imaging was performed 2 days prior to starting nelfinavir in 4 patients and 1 day before in 2 patients. The repeat imaging was performed on day 6 of nelfinavir therapy in 3 patients and day 7 in 3 patients. FMISO-PET imaging was acquired as per protocol in all patients at all time-points. The pCT was acquired as per protocol on all patients except Patient D. At the second imaging time-point the tumour was not contained within the field of view in this patient and could therefore not be assessed.

3.3.2.2 Quantifying hypoxia on the 4 h FMISO-PET/CT image

The 4 h FMISO-PET images were quantified and the results can be seen in Table 10. There was no significant difference in the SUV_{max}, SUV_{peak}, T:M_{max}, T:M_{peak}, T:B_{max} or T:B_{peak} from baseline to post-nelfinavir monotherapy. Median baseline values for SUV_{max}, SUV_{peak},

$T:M_{\max}$, $T:M_{\text{peak}}$, $T:B_{\max}$ and $T:B_{\text{peak}}$ were 2.1, 1.7, 1.7, 1.5, 1.7 and 1.4 respectively. Post-nelfinavir median values for $SUV_{\max}$, SUV_{peak} , $T:M_{\max}$, $T:M_{\text{peak}}$, $T:B_{\max}$ and $T:B_{\text{peak}}$ were 2.2, 1.7, 1.9, 1.5, 1.8 and 1.4 respectively. Figure 30 shows FMISO-PET images at baseline and repeat imaging in Patient D. On the magnified images of the tumour ROI, it can be seen that the maximum and peak values do not change significantly, but uptake throughout the volume is visually different.

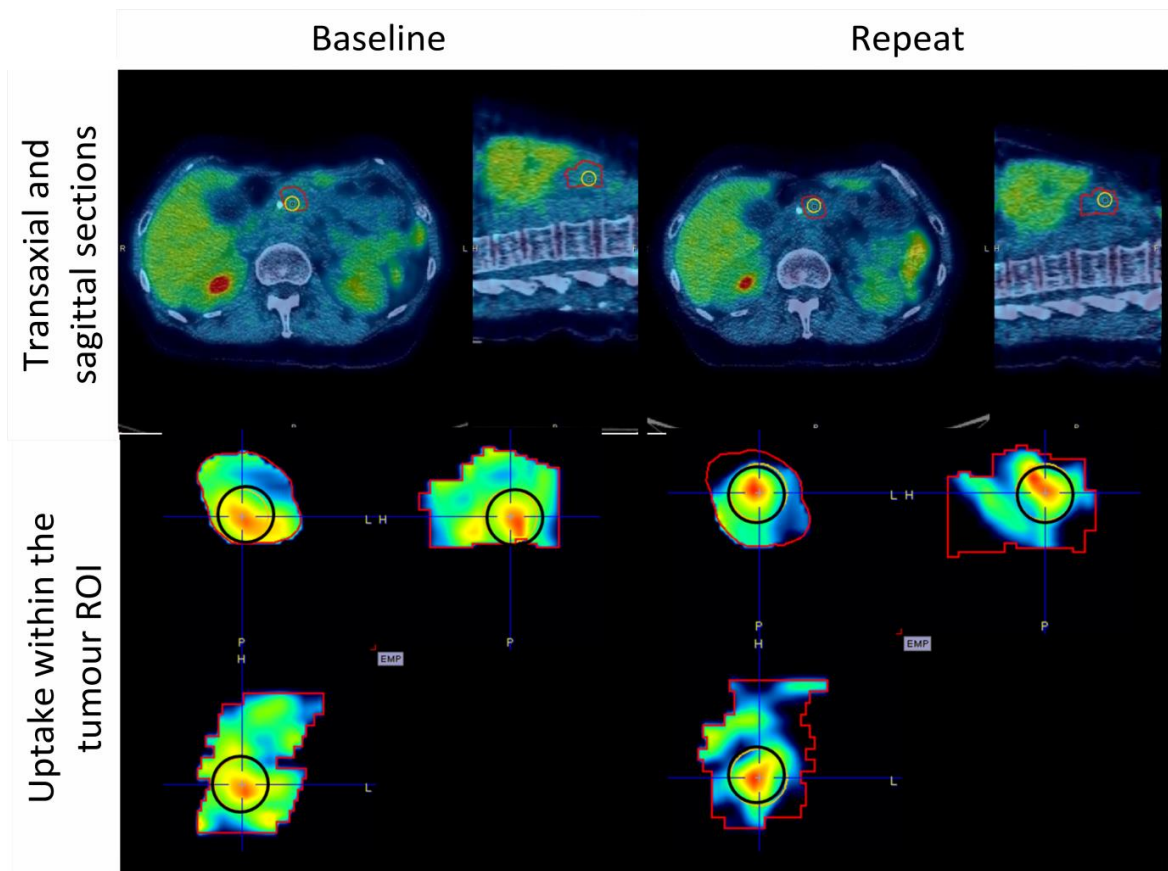


Figure 30: FMISO-PET/CT scans in Patient D at baseline and post-nelfinavir monotherapy. The tumour ROI is outlined in red. Transaxial and sagittal sections of the PET/CT can be seen with the GTV (red) and SUV_{peak} sphere. A magnified image of the tumour ROI is then presented with a detailed view of FMISO uptake within the tumour. The SUV_{peak} ROI can be seen to have shifted 5.6 mm from baseline to repeat imaging.

Table 10: Maximum and peak SUVs, T:M and T:B from FMISO-PET/CT acquired 4 h post tracer injection at baseline and when repeated after nelfinavir monotherapy.

	SUV _{max}		SUV _{peak}		T:M _{max}		T:M _{peak}		T:B _{max}		T:B _{peak}	
Patient	Baseline	Repeat	Baseline	Repeat	Baseline	Repeat	Baseline	Repeat	Baseline	Repeat	Baseline	Repeat
A	1.7	1.7	1.3	1.4	1.6	1.6	1.2	1.3	1.6	1.6	1.2	1.3
B	2.3	2.3	1.9	1.7	1.8	1.9	1.5	1.5	2.0	2.4	1.6	1.8
C	2.1	2.2	1.8	2.0	1.7	1.8	1.5	1.6	1.5	1.6	1.3	1.4
D	1.9	1.8	1.5	1.6	1.5	1.6	1.3	1.4	1.4	1.5	1.2	1.3
E	2.6	2.4	2.1	1.7	2.2	2.2	1.9	1.6	1.9	1.9	1.6	1.4
F	1.9	2.3	1.6	1.6	1.7	2.1	1.5	1.5	1.8	2.1	1.6	1.5
Median	2.1	2.2	1.7	1.7	1.7	1.9	1.5	1.5	1.7	1.8	1.4	1.4

Table 11: FMISO-derived 'hypoxic volumes'. Both absolute volumes (cm³) of FMISO uptake and as a percentage of the TV are presented. A T:M > 1.3 and T:B > 1.4 were used to define the hypoxic volumes.

Patient	Tumour Volume	Pre T:M 1.3 isocontour volume (cm ³)	Post T:M 1.3 isocontour volume (cm ³)	Pre T:M > 1.3 as % of GTV	Post T:M > 1.3 as % of GTV	Pre T:B 1.4 isocontour volume (cm ³)	Post T:B 1.4 isocontour volume (cm ³)	Pre T:B > 1.4 as % of GTV	Post T:B > 1.4 as % of GTV
A	20.8	0.3	0.4	1.5	1.8	0.1	0.2	0.5	0.8
B	23.5	6.0	5.3	25.4	22.4	6.3	7.5	26.8	32.0
C	34.9	14.4	16.9	41.3	48.4	0.1	0.6	0.3	1.6
D	11.3	0.9	3.2	7.7	28.0	0.0	0.1	0.0	1.1
E	13.5	9.3	11.3	68.7	83.4	2.4	5.7	17.5	42.0
F	34.0	3.0	8.4	8.8	24.6	3.0	3.4	8.9	10.1
Median	22.2	4.5	6.8	17.1	26.3	1.2	2.0	4.7	5.9

3.3.2.3 Defining 'hypoxic volumes' on the 4 h FMISO-PET/CT

All of the tumours demonstrated some evidence of hypoxia defined as a T:M > 1.3 or a T:B > 1.4. The uptake volume varied considerably between patients with uptake volumes varying from 1.5–83.4% of the TV when a T:M > 1.3 was used and 0.0–42.0% of the TV when a T:B > 1.4 was used to signify hypoxia. There was a trend for the hypoxic volume to increase from baseline to post-nelfinavir imaging. The change in volume was not statistically significant when a T:M > 1.3 was used ($p = 0.09$), but was significant when a T:B > 1.4 was used to define the hypoxic volume ($p = 0.03$). These results are summarised in Table 11.

3.3.2.4 The spatial consistency of FMISO uptake

The mean distance from the centre of the SUV_{peak} sphere at baseline to the centre of the SUV_{peak} sphere on the repeat testing was 6.0 mm (range 3.2–11.3 mm). The individual patient values can be seen in Table 12 and a representation of how the peak volumes spatially relate to one another in three dimensions can be seen in Figure 31.

Table 12: Characterising the change in position of the FMISO-PET SUV_{peak} from baseline to repeat imaging.

Patient	Shift in SUV _{peak} between time-points (mm)
A	6.4
B	11.3
C	5.2
D	5.8
E	3.8
F	3.2
Mean	6.0
Median	5.2

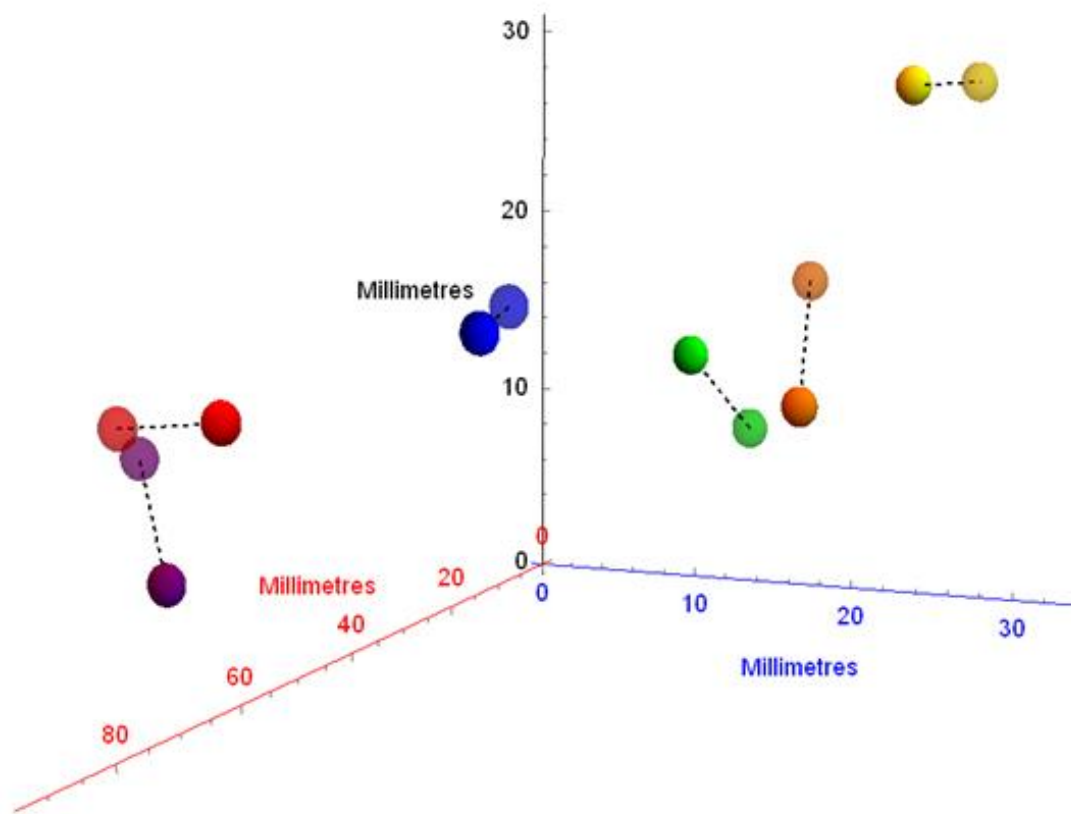


Figure 31: A representation of the geographical shift in position of the SUV_{peak} sphere at baseline and repeat imaging. The dotted line represents the measured distance between the central points of the SUV_{peak} spheres. Each patient is represented by a different coloured pair of spheres. The point (0,0,0) represents the normalised centre of mass of each tumour.

Baseline and repeat FMISO-PET imaging for Patient D can be seen in Figure 30. Both transaxial and sagittal sections of the FMISO-PET/CT can be seen and a magnified view of axial, sagittal and coronal sections of the tumour ROI. The centre of the SUV_{peak} can be seen to have shifted by 5.6 mm in an anterolateral direction.

3.3.2.5 Derivation of an arterial input function from image-derived and decay-corrected blood activity

It was possible to derive an AIF for all 12 of the FMISO-PET images. Although four blood samples were taken at each imaging time-point, the first (venous) sample had a decay-

corrected activity that was out of keeping with the image-derived blood activity by two orders of magnitude. This variable was therefore not included when fitting an AIF model using Dr Sara Hackett's software.²¹⁷ An example of how well the measured (image-derived and decay-corrected arteriovenous blood) activity can be seen in Figure 32.

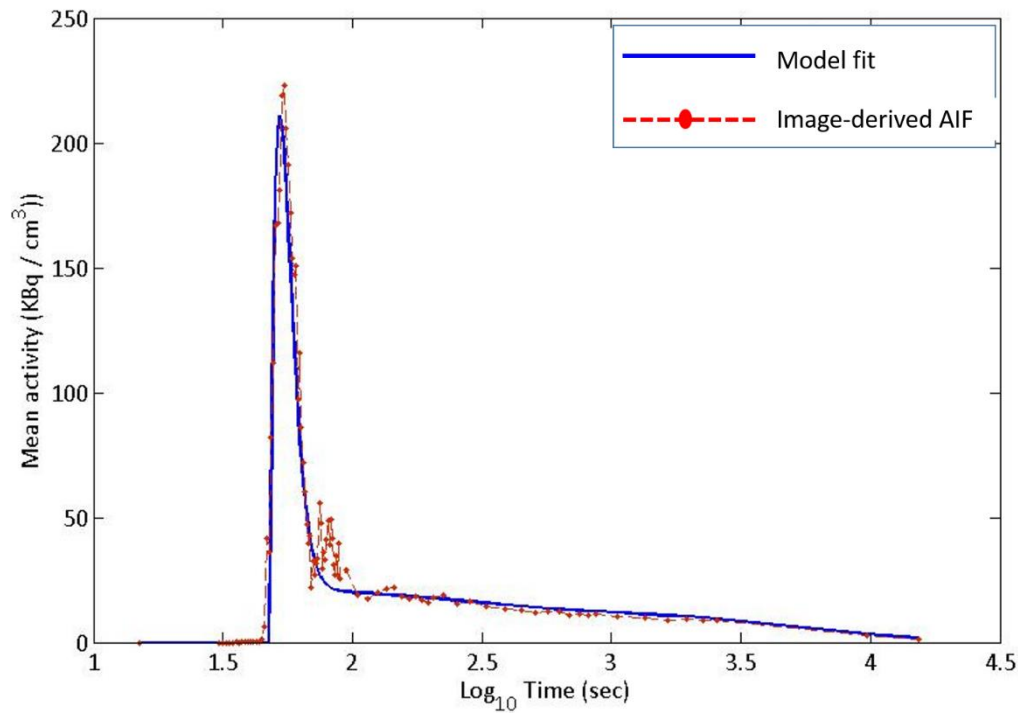


Figure 32: Measured blood activity (red) and fitted AIF (blue) for Patient A's baseline FMISO-PET.

3.3.2.6 Comparison of two-, three- and four-compartmental models in describing tumour

FMISO kinetics

The goodness of fit of the various models to the measured tumour TAC for the pre-nelfinavir FMISO-PET scan in Patient B is presented as an example in Figure 33. A comparison of how well the various models fit each of the FMISO-PET images using the runs test, AIC and BIC can be seen in Table 13. The 2C4K model passes the runs test most frequently, in 6 of 12 cases. Where possible, the simplest model that can be used to

describe FMISO kinetics should be adopted. The AIC/BIC values for the 2C4K model are comparable with the values seen with the three-compartmental models. The 2C4K was therefore used for all parameter quantification.

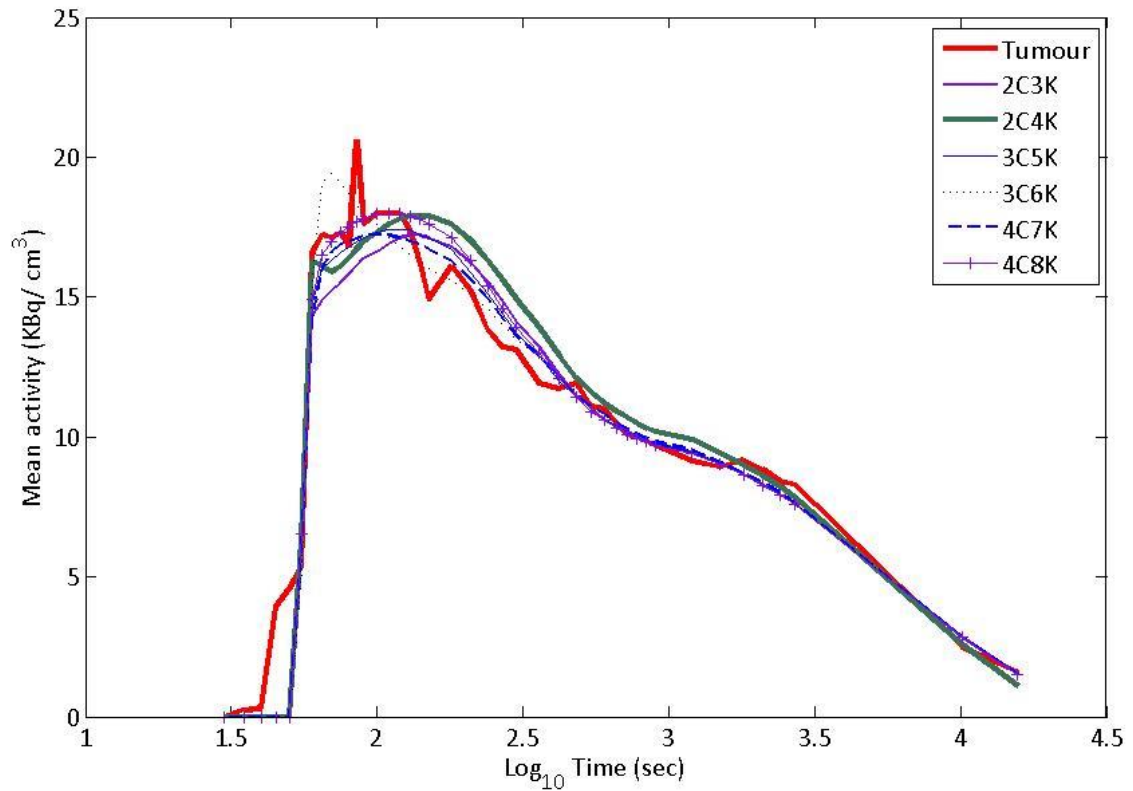


Figure 33: Demonstration of model fit to the measured tumour TAC for dynamic FMISO-PET in Patient B at baseline. The measured tumour TAC can be seen in red. The closest fit to the data as assessed by Akaike and Bayesian information criteria was the 2C4K model (green).

2C3K = two-compartment, three-parameter model, 2C4K = two-compartment, four-parameter model, 3C5K = three-compartment, five-parameter model, 3C6K = three-compartment, six-parameter model, 4C7K = four-compartment, seven-parameter model, 4C8K = four-compartment, eight-parameter model (see Figure 22).

Table 13: Comparing the two-, three- and four-compartment models' ability to describe observed FMISO TACs. Akaike information criteria (AIC), Bayesian information criteria (BIC) and number of passed runs test for the model fit to measured data is presented.

	AIC/BIC* for 2, 3 and 4 tissue models					
Patient/ Image set	2C4K	2C3K	3C6K	3C5K	4C8K	4C7K
A-Pre	320.4/327.4	494.5/500.4	319.3/328.1	316.2/324.3	322.4/331.9	322.4/331.9
A-Post	332.9/339.9	494.5/500.4	348.9/357.8	338.8/346.8	356.3/366.3	341.4/351.0
B-Pre	368.9/376.0	358.5/364.3	358.4/367.3	359.3/367.3	370.6/380.6	371.2/379.8
B-Post	350.4/357.5	361.2/367.1	320.6/329.5	350.4/358.4	367.9/377.9	388.9/398.4
C-Pre	349.1/356.2	493.5/499.4	348.2/357.1	348.2/357.1	348.2/357.1	346.1/355.7
C-Post	347.2/354.2	545.1/551.0	342.0/350.8	350.6/358.6	359.9/369.9	359.9/369.9
D-Pre	344.9/352.0	538.9/543.9	350.6/359.5	347.8/355.8	356.1/366.1	352.6/362.2
D-Post	372.6/379.6	521.2/527.1	377.2/386.1	374.0/382.0	383.2/393.3	380.5/390.0
E-Pre	390.4/397.4	552.5/558.4	346.4/355.3	350.5/358.5	402.4/412.4	356.4/366.0
E-Post	400.4/407.4	509.6/515.5	448.1/457.0	445.3/453.3	451.2/460.8	451.2/460.8
F-Pre	340.5/347.5	427.3/433.1	346.5/355.4	343.8/351.9	333.8/343.8	350.6/360.1
F-Post	357.4/364.4	359.6/365.4	358.9/367.8	356.4/364.5	363.2/373.3	360.0/369.5
Runs test passed	6/12	0/12	4/12	2/12	2/12	1/12
Total AIC	4275.1	5656.4	4265.1	4281.3	4415.2	4381.2
Total BIC	4395.5	5726	4371.7	4378.5	4533.4	4115.5

3.3.2.7 Changes in the intracellular retention of FMISO

Fitting the 2C4K model to the image-derived TACs allowed the dynamic parameter values to be quantified, the results of which can be seen in Table 14. The k_3 , which describes FMISO intracellular retention, was shown to decrease in 4 of the 6 patients and rise in the remaining 2 patients. Overall, the k_3 value decreased a mean and median of 24.5% and 58.7% respectively. In those whose k_3 improved, the mean decrease was 75.3 % (range 50–97%). In the 2 patients in whom the k_3 increased, the mean increase was 77.0% (range 75.3–78.6%).

Table 14: Parameter values derived from the application of a 2C4K model to FMISO-PET tumour TACs and the change in k_3 , which describes FMISO retention.

	Pre-nelfinavir				Post-nelfinavir				
Patient	K_1	k_2	k_3	k_4	K_1	k_2	k_3	k_4	$\% \Delta k_3$
A	0.059	0.400	0.056	0.013	0.072	1.400	0.100	0.007	78.6
B	0.052	0.760	0.170	0.016	0.014	0.029	0.005	0.005	-97.0
C	0.110	1.900	0.097	0.011	0.027	1.700	0.170	0.005	75.3
D	0.026	0.500	0.260	0.013	0.041	0.440	0.130	0.012	-50.0
E	0.008	0.400	0.460	0.010	0.019	0.460	0.150	0.006	-67.4
F	0.034	0.580	0.082	0.008	0.010	0.027	0.011	0.006	-86.6
Mean									-24.5
Median									-58.7

3.3.2.8 Correlation of k_3 with 4 h FMISO-PET-derived parameters

There was no significant correlation between the k_3 parameter values derived from a 2C4K model applied to the dynamic FMISO-PET data and uptake values derived from the 4 h FMISO-PET image. Spearman's rho of the correlation and the significance (P) of the correlation between k_3 and 4 h FMISO-PET with SUV_{max} , SUV_{peak} , $T:M_{max}$, $T:M_{peak}$, $T:B_{max}$ and $T:B_{peak}$ can be seen in Table 15.

Table 15: Assessing the correlation of tumour retention as described by k_3 from a 2C4K model and uptake seen on FMISO-PET imaging 4 h post FMISO injection.

	Correlation of k_3 with:					
	SUV _{max}	SUV _{peak}	T:M _{max}	T:M _{peak}	T:B _{max}	T:B _{peak}
Spearman's rho	0.255	0.438	0.021	0.326	-0.329	-0.123
P	0.424	0.154	0.948	0.301	0.296	0.703

3.3.3 Results of the pCT analysis

Relative change in BF and BV was only available in 5 of the 6 patients. The repeat pCT in one of the patients (Patient D) did not adequately image the pancreatic tumour as the field of view was set up incorrectly. BF, BV and relative change in BF and BV for all the patients can be seen in Table 16. A wide range of responses in BF and BV was seen across patients.

3.3.3.1 Non-motion-corrected imaging

The mean change in BF seen on the non-motion-corrected pCT was 15.2%, 14.7% and 21.4% for the whole tumour, tumour core and tumour periphery respectively. For BV, the mean change was 0.8%, -8.2% and 7.3% for the whole tumour, tumour core and tumour periphery (see Figure 34). Apart from the peripheral zone of the tumour in Patient C, the direction of change in BF was consistent. The direction of change in BV was consistent, irrespective of the tumour region that was being assessed in all patients.

3.3.3.2 Motion-corrected imaging

When the motion-corrected pCTs were analysed, the mean change in BF was 8.8%, 3.5% and 14.7% for the whole tumour, tumour core and tumour periphery respectively. The mean change in BF was 11.8%, 26.7% and 14.9% for the whole tumour, tumour core and tumour periphery (see Figure 34). The direction of change in BF was consistent across all

tumour regions apart from the peripheral zone of the tumour in Patient C. The direction of change in BV was more variable, with only Patient B demonstrating the same direction of change in all tumour regions. The other patients showed disagreement in the direction of change in at least one of the parameters.

3.3.3.3 Comparing parameters from unprocessed and motion-corrected imaging

The median and range of BF and BV values when derived from unprocessed and motion-corrected pCT images can be seen in Table 16. The only statistically significant difference was seen in BF in the tumour periphery, which is most likely to be because some outlying data points seen in the unprocessed pCT analysis were reduced by motion correction.

As illustrated in Figures 34 and 35 and Table 17, the magnitude and direction of change in BF and BV was not consistent in individual patients when unprocessed image values were compared with those derived from motion-corrected images. While the median values were not significantly different in the entire cohort, the percentage change from pre- to post nelfinavir was not consistent when comparing parameter values from unprocessed and motion-corrected imaging on an individual basis. Examples of the percentage change in BF and BV values for the whole tumour volume using the unprocessed and motion-corrected images can be seen in Figure 35.

Table 16: A comparison of BF and BV values when derived from unprocessed and motion-corrected pCT images. Values were compared using the Wilcoxon matched-pairs signed-rank test.

		Unprocessed	Motion-corrected	P
BF (mL/100 g/min) Median (range)	Whole tumour	43.7 (30.0–70.5)	37.9 (29.0–55.9)	0.123
	Core	37.3 (27.7–58.0)	36.5 (21.7–51.5)	0.320
	Periphery	37.3 (32.2–71.1)	36.5 (34.9–57.3)	0.032
BV (mL/100 g) Median (range)	Whole tumour	5.9 (3.4–8.4)	5.0 (3.7–7.1)	0.156
	Core	5.4 (2.6–7.4)	4.3 (2.9–7.9)	0.432
	Periphery	5.9 (4.0–9.2)	5.7 (4.3–8.2)	0.919

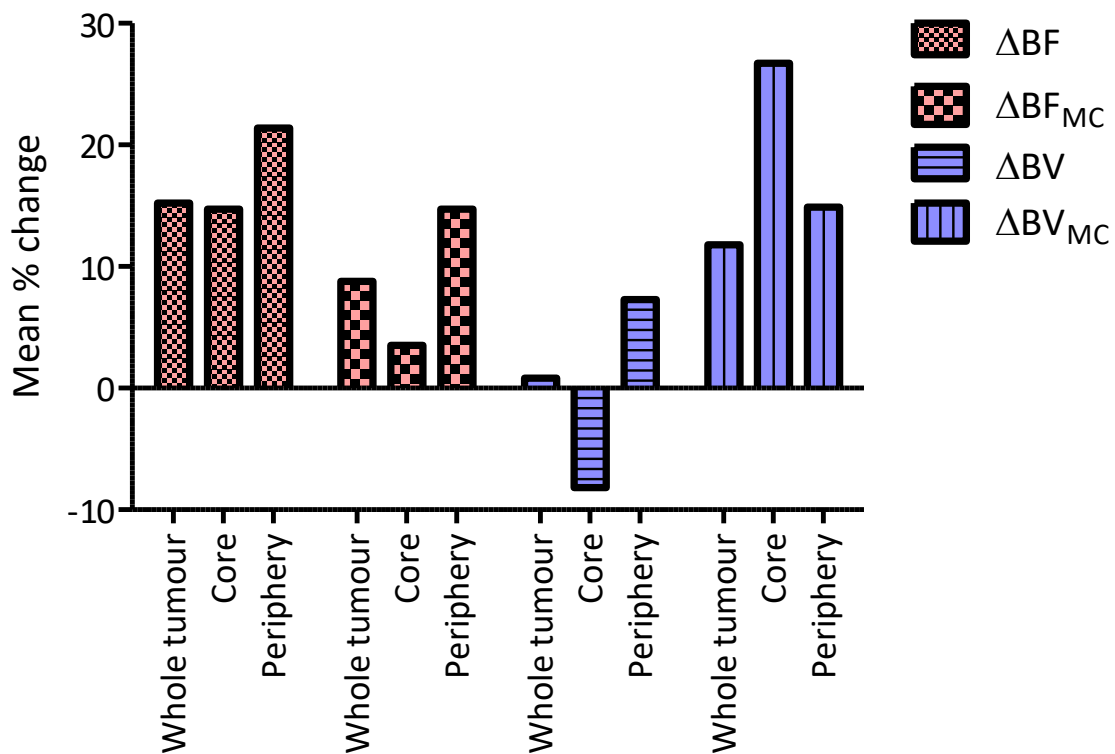


Figure 34: Mean change in BF and BV as assessed by pCT. BF and BV were quantified for the whole tumour, the tumour core and the tumour periphery. Both non-motion-corrected and motion-corrected (MC) imaging was analysed.

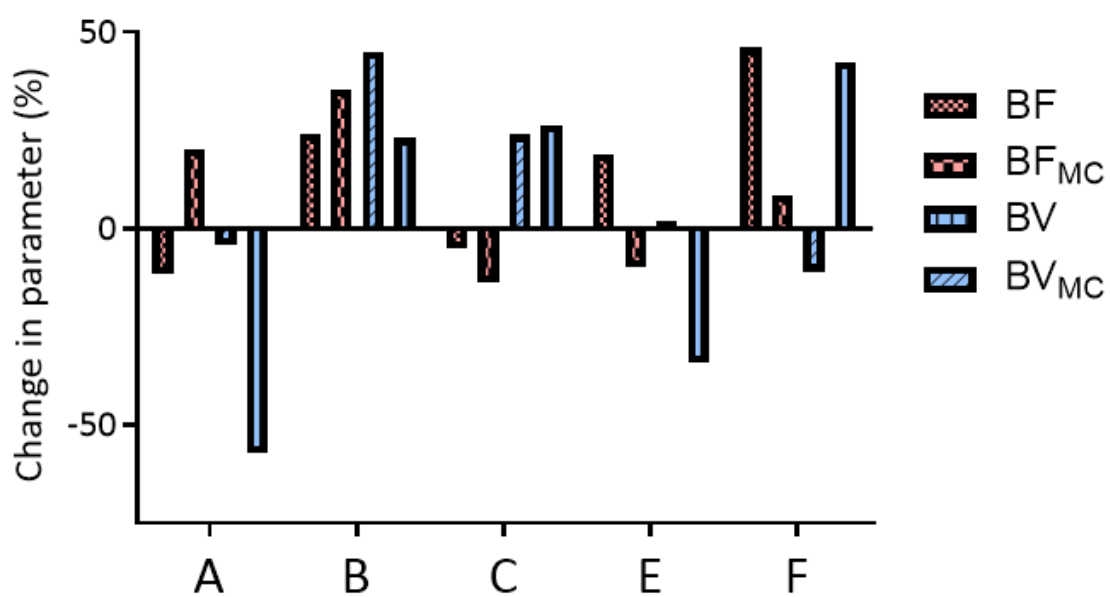


Figure 35: Comparing the direction of change in BF and BV derived from the whole tumour volume from baseline to post-nelfinavir imaging depending on whether the unprocessed or motion-corrected (MC) imaging was analysed.

Table 17: Perfusion CT-derived BF and BV values for the whole tumour, tumour core and tumour periphery. Values for unprocessed pCT data analysis and motion-corrected imaging is presented.

	Patient	Whole tumour						Core						Periphery					
		BF			BV			BF			BV			BF			BV		
		Pre-	Post-	%Δ	Pre-	Post-	%Δ	Pre-	Post-	%Δ	Pre-	Post-	%Δ	Pre-	Post-	%Δ	Pre-	Post-	%Δ
Non-motion-corrected	A	48.4	43.7	−9.7	7.6	3.4	−55.3	44.4	40.3	−9.2	6.7	2.6	−61.2	50.3	44.9	−10.7	8.0	3.7	−53.8
	B	37.1	46.0	24.0	6.1	7.5	23.0	31.8	37.3	17.3	5.4	6.0	11.1	41.2	52.5	27.4	6.7	8.5	26.9
	C	36.7	35.5	−3.3	3.8	4.8	26.3	33.6	27.7	−17.6	3.6	4.3	19.4	39.8	44.1	10.8	4.0	5.3	32.5
	D	70.5			5.7			58.0			4.7			71.1			5.9		
	E	40.5	48.2	19.0	6.2	4.2	−32.3	41.2	46.9	13.8	6.6	3.8	−42.4	39.9	49.2	23.3	5.8	4.5	−22.4
	F	30.0	43.8	46.0	5.9	8.4	42.4	21.4	36.2	69.2	5.6	7.4	32.1	32.2	50.3	56.2	6.0	9.2	53.3
	Mean	43.9	34.4	15.2	5.9	5.7	0.8	38.4	37.7	14.7	5.4	4.8	−8.2	45.8	48.2	21.4	6.1	6.2	7.3
Motion-corrected	A	37.7	45.3	20.2	4.2	4.1	−2.4	34.2	40.0	17.0	2.9	3.4	17.2	40.5	45.3	11.9	4.5	4.3	−4.4
	B	31.8	43.1	35.5	4.9	7.1	44.9	26.9	36.5	35.7	4.0	7.9	97.5	34.9	49.0	40.4	5.5	8.5	54.5
	C	37.9	33.4	−11.9	4.2	5.2	23.8	36.8	27.9	−24.2	3.7	4.6	24.3	39.0	41.5	6.4	4.7	6.2	31.9
	D	55.9			3.7			51.5			4.7			57.3			8.2		
	E	31.6	29.0	−8.2	5.5	5.6	1.8	23.4	21.7	−7.3	4.3	4.3	0.0	38.3	35.6	−7.0	6.5	6.8	4.6
	F	41.2	44.7	8.5	5.5	5.0	−9.1	43.3	41.6	−3.9	5.2	4.9	−5.8	39.4	48.0	21.8	5.7	5.0	−12.3
	Mean	39.4	39.1	8.8	4.7	5.4	11.8	36.0	33.5	3.5	4.1	5.0	26.7	41.6	43.9	14.7	5.9	6.2	14.9

3.3.3.4 Differences between changes in perfusion at the tumour core and tumour periphery

In the unprocessed pCT imaging, a larger change in BF and BV was consistently seen in the tumour periphery compared with the tumour core. When motion correction had been applied to the images, an increase in BF was observed in the tumour periphery compared with the tumour core, but the opposite was true for BV. Correcting the image for motion appears to decrease the increase in perfusion observed in the tumour periphery (mean 3.5% compared with 14.7% in the unprocessed pCT).

3.3.3.5 Inpatient heterogeneity in changes in BF and BV

A wide range in changes in BF and BV was observed between patients in both the non-motion-corrected and motion-corrected images. This heterogeneity is shown in Figure 36. For the unprocessed imaging, the range of response in BF from pre-treatment to post nelfinavir was -9.7% to 46.0%, -17.6% to 69.2%, -10.7% to 56.2% when viewed over the whole tumour, the tumour core and tumour periphery respectively. The range of response in BF for the motion-corrected imaging was from -11.9% to 35.5%, -24.2 to 35.7% and -7.0% to 40.4% in the whole tumour, core and periphery. The direction of change in BF was positive (an increase in BF) in 3 out of 5 patients when the whole tumour was taken into account in both the unprocessed and motion-corrected images. There was, however, no agreement on an individual patient basis: one patient that demonstrated a decrease in BF in the unprocessed images had an increase in the motion-corrected images (Patient A). Conversely, Patient E had an increase in BF on the unprocessed images and a decrease when motion correction was added. Only Patients B

and C had consistency in the direction of change in BF in all tumour regions (whole, core or periphery) in both unprocessed and motion-corrected imaging.

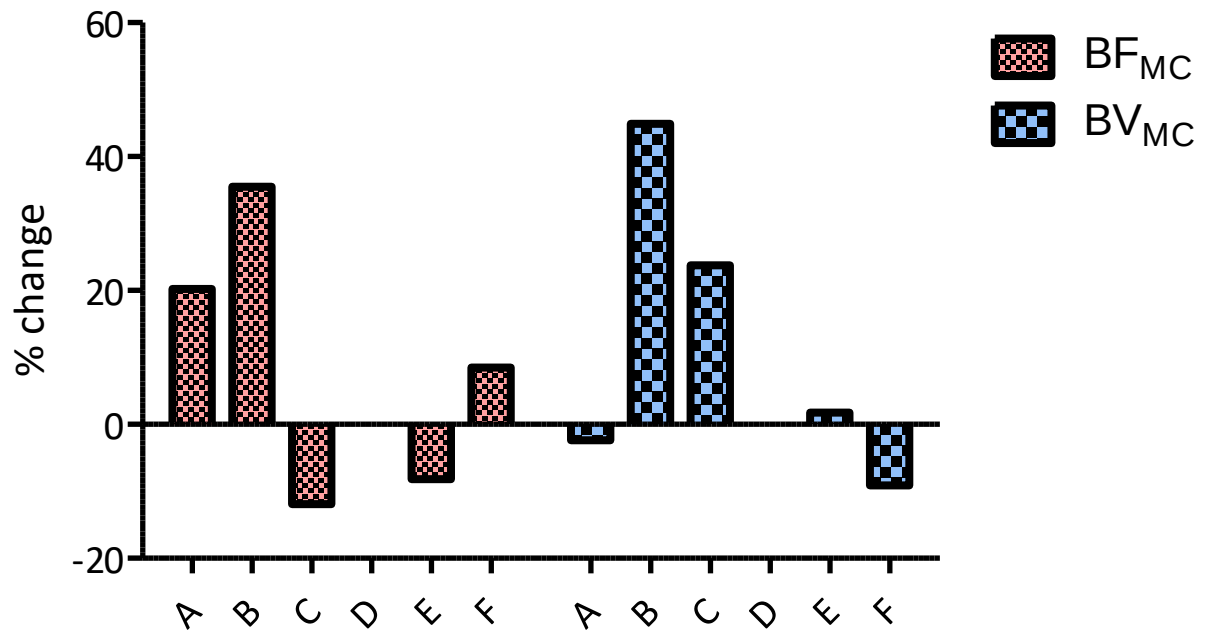


Figure 36: Changes in motion-corrected (MC) pCT-derived BF and BV. The range of response of BF and BV were from -11.9% to 35.5% and from -9.1% to 44.9% respectively.

3.3.3.6 Correlating changes in hypoxia with blood flow

Individual changes in dynamic FMISO-PET FMISO retention were quantified using k_3 from a 2C4K model. Changes in k_3 observed in each patient are listed in Table 14. As this was quantified across the whole tumour ROI, it was plotted against the changes in BF across the whole tumour (both the unprocessed and motion-corrected pCT) in Figure 37. The 'improvement' in the parameter has been plotted. This means that a reduction in k_3 and an increase in BF would be plotted as a positive integer. For example, Patient B had a reduction in k_3 of 97%, so appears on the histogram as an 'improvement' of 97%, while Patient A had an increase in k_3 of 78.6% which appears on the histogram as a negative 'improvement' of -78.6%.

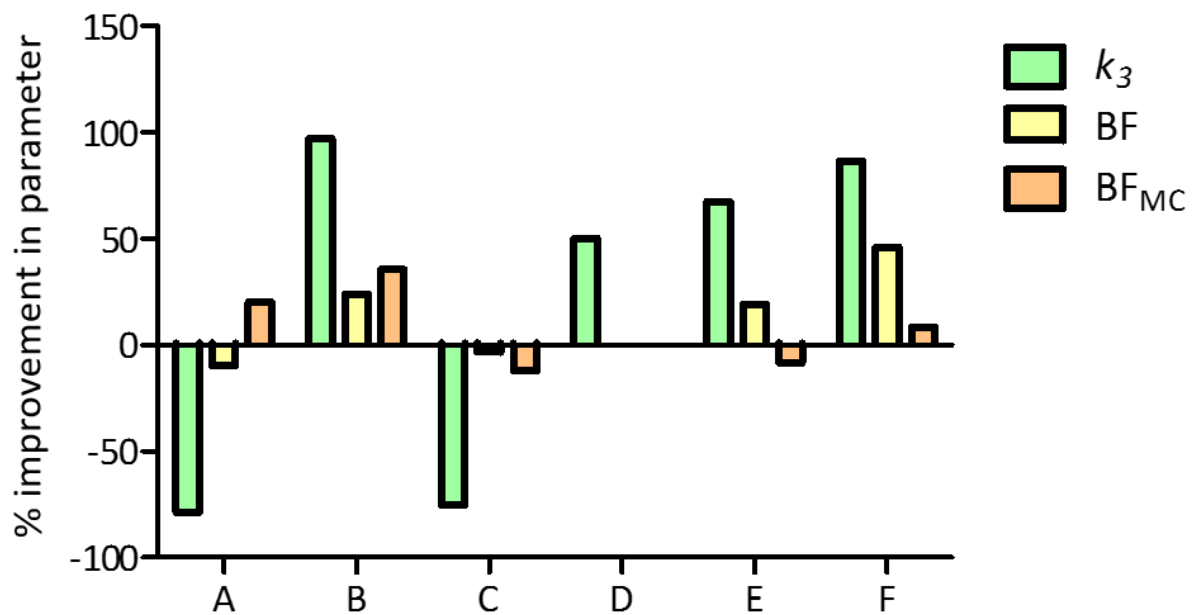


Figure 37: Changes in dynamic-FMISO-PET k_3 (representing FMISO retention) and pCT BF across the whole tumour (both unprocessed and motion-corrected [MC] parameter values presented) for each of the 6 patients (A–F).

When the unprocessed pCT image-derived BF values are used, the direction of change in the hypoxia and perfusion imaging is consistent in all of the evaluable patients. When the motion-corrected pCT BF values are used, this falls to an agreement in only 3 of the 5 patients.

In the 4 patients who had a reduction in k_3 , the mean % change in the whole tumour BF (derived from non-motion-corrected images) was 58.3% compared with a 6.5% decrease in mean BF in those who did not have an improvement in FMISO-PET k_3 .

3.4 Discussion

Delivering an explicitly non-uniform radiotherapy dose distribution, such as boosting a biological subvolume, to improve rates of local tumour control is the subject of much interest. Treatment outcomes in LAPC are poor and approximately 1 in 4 patients have the

initial site of treatment failure within the GTV.^{21,22} The work in this chapter describes two imaging modalities that could be used to define a BTV for radiotherapy dose escalation: FDG-PET and FMISO-PET. FMISO-PET and pCT have also been used to characterise changes in tumour hypoxia, quantified by FMISO retention on dynamic FMISO-PET analysis, and pCT-derived BF following treatment with the Akt inhibitor nelfinavir.

3.4.1 Using FDG-PET imaging to define a BTV for radiotherapy dose escalation

FDG-PET may be useful in identifying a BTV. The results of this study suggest that areas of residual metabolic activity following CRT can be predicted by looking at volumes of $\geq 40\%$ of SUV_{max} on the pre-CRT FDG-PET scan. This supports the hypothesis that areas of increased FDG uptake prior to treatment have a degree of chemo- and radioresistance that had been suggested from preclinical⁹⁸ and rectal cancer data.¹⁰⁰ Models of tumour heterogeneity suggest that the optimal dose prescription can be approximated using only a few dose levels – if the compartment boundaries and prescribed dose levels are well chosen.²²²

The Pre40% FDG-PET volume seems to be the most pragmatic to use for BTV definition. It covers most of the residual FDG-avid areas and only covers an average of 50.8% of the GTV, so dose escalation without increasing toxicity to normal tissues may be feasible. The Pre50% volume has been recommended as a suitable boost volume in NSCLC as the Pre40% volume covered almost all of the tumour⁹⁹; the authors proposed that, a mean overlap fraction between the Pre50% and the Post90% volume of 70.4% supported the use of the Pre50% volume for BTV definition. In our study, the mean overlap fraction of the Pre40% volume with the Post90% volume was 83.3%, with 100% of the volume being included in all but 2 patients while covering an average of only 50.3% of the GTV.

Absolute SUVs for volume delineation (data not shown) were also applied on the pre- and post-CRT FDG-PET images. Using an SUV value of 2.5, it was difficult to differentiate from the metabolic activity in adjacent normal tissues – most noticeably in the liver and small bowel. It was therefore decided that values relative to the SUV_{max} should be used to segment the tumours. A number of thresholds have been used to segment tumours of various types to aid target volume definition based on FDG-PET SUV_{max}. The percentages most commonly used are 40% and 50% of the SUV_{max} (reviewed in Biehl *et al.*²²³). These values were therefore used in this study. Another study used values from 34% to 70% of the SUV_{max} on the pre-treatment FDG-PET to segment the tumour.⁹⁹ Values lower than 40% of the SUV_{max} produced large volumes that extended beyond the region of interest and that using a threshold $\geq 60\%$ of SUV_{max} produced a volume that did not sufficiently overlap with areas of residual metabolic activity. Post-CRT thresholding was done at levels $\geq 60\%$. As the SUV_{max} had decreased during CRT segmentation, using a threshold $< 60\%$ of SUV_{max} led to the inclusion of normal tissue in the volume. The smaller uptake volume on the post-CRT FDG-PET will make the SUV appear reduced due to the recovery coefficient of the scanner. This may also explain why higher relative thresholds were required to produce volumes that were contained by the residual tumour mass on the post-CRT imaging. It could be that a gradient-based segmentation method, as discussed in Chapter 1, may be a more appropriate means of delineating the target volumes so that the impact of partial volume effect does not need to be taken into account.

The commercially available non-rigid registration algorithm employed to relate post-CRT FDG uptake with pre-CRT FDG-PET-derived volumes has been validated for a number of clinical tumour sites²²⁴⁻²²⁶ and been shown to give residual registration errors typically less

than 3 mm, depending on the clinical site and the magnitude of initial displacement. All registrations were checked visually by a second expert radiation oncologist.

The change in both the size and location of the FDG-PET-defined target further highlights the need for adaptive planning during radiotherapy for LAPC – particularly if a dose boost to a metabolically active subvolume is to be considered. Further investigation into how the tumour shrinks throughout treatment, perhaps with functional imaging with adaptive thresholding, may increase confidence in the location of the BTV within the GTV.

3.4.2 Using FMISO-PET to characterise hypoxia in LAPC

To my knowledge, this is the first report of the successful characterisation of hypoxia in the pancreas on sequential FMISO-PET imaging. The only previous report on FMISO-PET in pancreatic cancer did not support its ongoing use in this clinical setting. By applying a rigorous methodology gleaned from studies in other tumour sites, I was able to demonstrate evidence of tumour hypoxia in all 12 of the FMISO-PET studies. In the only published series in pancreatic cancer performed the FMISO-PET scan was performed 2 h post FMISO injection.⁹⁴ The findings in this study support delaying imaging until 4 h post FMISO injection. It has been observed in other tumour sites that the signal-to-background ratio was too low before 4 h.^{95,96,153}

The signal-to-background ratio used to quantify hypoxia in the previously published pancreatic series was not stated. The thresholds used to signify hypoxia in this study can be justified from both the preclinical and clinical literature. When FMISO activity in the normal tissues of four animal species after FMISO scanning was pooled and quantified, the mean FMISO normal tissue-to-blood ratio was essentially unity and 90% of the tissue-to-blood-ratios were <1.3 .¹⁵³ This led early investigators to choose a $T:B \geq 1.4$ to represent

significant hypoxia within the ROI.¹⁵³ This was validated in human studies by demonstrating that the mean FMISO muscle-to-blood ratio was 0.98, with 87% of pixels in normal tissue having a tissue-to-blood ratio ≤ 1.4 .¹⁷⁷ Applying a T:M >1.4 has therefore been adopted in a number of clinical series.^{95,170-172}

By applying thresholds based on tumour uptake-to-background ratios to the image acquired 4 h post FMISO injection, it was possible to demonstrate tumour hypoxia on all 12 of the scans – this is much more in keeping with the data from operable pancreatic cancers which demonstrated evidence of hypoxia in the majority of pancreatic tumours.^{227,228}

While a correlation was not observed between tumour k_3 values and maximum and peak values seen on the 4 h FMISO-PET, this could be explained by k_3 being derived from the whole TV, while maximum values are a single-pixel value and peak values represent the activity in the 1 cm³ sphere with the highest mean activity. Derivation of k_3 values from only the 1 cm³ peak volume may demonstrate a closer correlation and support the use of k_3 from a 2C4K model to describe tumour hypoxia.

3.4.3 Defining a 'hypoxic' BTV for radiotherapy dose escalation using FMISO-PET

Areas of peak FMISO uptake 4 h post tracer injection within the pancreatic tumours were geographically and quantitatively stable suggesting the presence of regions of chronic hypoxia. The use of FMISO-PET to derive hypoxic target volumes for radiotherapy dose boosting therefore shows promise. There was no statistical or clinical difference in maximum and peak uptake values on the sequential 4 h FMISO-PET/CTs despite nelfinavir therapy. The 10 min PET acquisition 4 h post FMISO injection was not a 4D acquisition, meaning that imaged activity will be time-averaged across the respiratory cycle. Random

set-up errors, even when taking care to replicate the radiotherapy treatment position, can be up to 3.2 mm in pancreatic tumours.²²⁹ A mean distance between the centre of the SUV_{peak} sphere on the two FMISO-PETs of 6.0 mm (range 3.2–11.3 mm) suggests that the region of chronic hypoxia in the tumour is therefore geographically consistent. As the spatial resolution of PET is limited by the physics of positron emission and scanner performance to approximately 4–5 mm, it can be concluded that the areas of most intense hypoxia are in the same region of the tumour.

The T:M and T:B defined 'hypoxic' volumes were smaller than the GTV, but large enough for dose escalation. In a planning study in head and neck cancer, FMISO-PET-defined hypoxic volumes ranged from 0.1 cm³ to 40.6 cm³, which represented 0.5–56.0% of the GTV. The investigators attempted to dose escalate to all of the hypoxic volumes in this series.⁹⁵ The FMISO-PET-derived hypoxic volumes observed in this study varied depending on the threshold used to define hypoxia and the imaging time-point. The largest hypoxic volumes were observed using a T:M > 1.3 at the second imaging time-point. The mean FMISO uptake volume was 26.3% (range 1.8–83.4%) of the GTV. The smallest hypoxic volumes observed were using a T:B > 1.4 at the first imaging time-point, where the mean volume was 4.7% (range 0.0–26.8%) of the GTV. These volumes are significantly smaller than the GTV and therefore could potentially be used to define a BTV. Before this approach could be adopted clinically, some inconsistencies observed in this small cohort of patients require investigation to decide how to best define a BTV using FMISO-PET.

A trend for the hypoxic volume to increase in size between time-points was observed. The increase in the T:B defined volume was statistically significant. For most of the patients, the increase in size of the FMISO uptake volume is unlikely to be clinically significant, but

for 2 patients, the hypoxic volume doubled (T:M volumes in Patient D, T:B volumes in Patient E). The test-retest variation in FMISO uptake volumes in pancreatic cancer, ideally without the use of nelfinavir between scans, requires further investigation. A spatial correlation analysis similar to that carried out for FDG-PET images in this chapter, would further define the consistency of the FMISO uptake distribution. It could be that addition of a margin to a baseline definition of a hypoxic BTV would adequately account for uncertainty in the uptake volume between scans.

The observed FMISO uptake volume could be quite small – particularly when a T:B was used to define significant uptake. When uptake volumes are small, PVEs should also be taken into account. The spatial resolution of PET is limited and volumes smaller than approximately 2.5 times the full width at half maximum will have an artificially reduced uptake value unless a partial volume correction is applied.⁶⁶ It would be interesting to see if correcting for PVE would alter the observed uptake volume and increase confidence in an FMISO-PET-derived hypoxic BTV.

The most robust method of using FMISO-PET to define a BTV is not yet clear. The most reliable reference tissue to use and the uptake threshold above background that most accurately defines hypoxia on static FMISO imaging has not been defined for pancreatic tumours. It is not yet known if the 4 h FMISO-PET uptake should be used or if dynamic FMISO parameter (e.g. k_3) maps would more accurately describe regional tumour hypoxia. A first step in answering this question might be to perform a voxel-wise analysis to relate 4 h imaging to kinetic parameter maps. This may also help understand why the global k_3 values did not correlate with maximum and peak values seen at 4 h. Kinetic parameter maps are noisy. It is, however, possible to identify PET voxels with similar uptake

characteristics based on BIC and expectation-maximisation clustering.²³⁰ In the case of hypoxia, only two distinct voxel clusters would need to be identified – hypoxic and normoxic. A means of validating the hypoxic volumes derived from either the 4 h FMISO-PET or from clustering of the parameter maps against histology of hypoxia markers would increase confidence in the ability of FMISO-PET to define hypoxic subregions of pancreatic tumours for dose boosting.

3.4.4 Characterising hypoxia and perfusion modulation by nelfinavir

3.4.4.1 Nelfinavir-modulated tumour hypoxia

Dynamic FMISO-PET adds further information to static 4 h FMISO-PET image quantification. It was possible to demonstrate a reduction in FMISO retention (k_3), and therefore hypoxia, from the baseline to the post-nelfinavir imaging time-point.

While there is some inherent uncertainty in the absolute k_3 values, some conclusions can probably be drawn from the direction of change in k_3 – particularly as the smallest change in the parameter from baseline to repeat scanning after nelfinavir monotherapy was a reduction of 50%. A reduction in k_3 was seen in 4 out of 6 patients.

No change was detected in maximum and peak parameters from the 4 h FMISO-PET images taken pre- and post nelfinavir. It could be concluded that there is a region of chronic hypoxia that is not altered by 6–7 days of nelfinavir, but regions of transient hypoxia are altered. This remains a hypothesis that needs to be tested, but it may explain the changes in the kinetic parameters in 4 out of 6 patients that are not mirrored in maximum and peak uptake parameter changes. As demonstrated in Figure 30, the uptake within the small volume SUV_{peak} sphere does not appear to change, but the uptake within the remaining TV

is altered. It could be this hypoxia modulation outside of the most intense hypoxia that is being described by the change in k_3 . Intracellular oxygen levels also exist on a continuum from well oxygenated cells to severe hypoxia depending on cellular consumption and distance from blood vessels. It might be that nelfinavir can reduce hypoxia in the minimally hypoxic cells in quite a short time period, but a longer therapy duration would be required before it impacts upon regions of severe, chronic hypoxia. This would fit with the observed difficulty some drugs have in entering pancreatic tumours because of their poor perfusion and high hydrostatic pressure.^{211,212} A voxel-wise analysis may demonstrate heterogeneity in hypoxia modulation across the TV.

Use of a two-compartmental model to describe FMISO tumour kinetics seems a reasonable approach as it was the simplest model that most accurately described the observed tumour TACs. A two-compartment model is also in keeping with our understanding of FMISO physiology. As we think that binding of FMISO in hypoxic cells is irreversible, it was perhaps surprising that reducing k_4 to zero when fitting the data to a two-compartmental model led to a worse fit. This could be explained by the slow movement of FMISO metabolites towards the end of the 4 h imaging period. This phenomenon has been observed in *in vitro* studies²³¹ and glioblastoma multiforme.¹⁶³ When applying the 2C4K model to this data, reducing k_4 to zero led to a worsening of model fit (as demonstrated by increasing AIC/BIC values, see Table 13). While not restricting k_4 improved the model fit, it can be seen in Table 14 that k_4 values were very small. This would be in keeping with only a small proportion of FMISO metabolites returning to the circulation towards the end of the 4 h scanning period.

3.4.4.2 *The effect of nelfinavir on tumour perfusion*

When changes in the unprocessed pCT BF values are plotted with changes in dynamic FMISO-PET k_3 values, there is agreement in the presence (4/6 patients) or absence of improvement in hypoxia and perfusion as a result of nelfinavir therapy (see Figure 37). This might have been expected, as a significant negative correlation has been observed between FMISO-PET parameters and another measurement of perfusion, dce-MRI, in head and neck lymph nodes.¹⁷⁵ While the pCT-derived parameter values seen in this study are comparable with other published series,²⁰⁸⁻²¹⁰ the level of uncertainty in the BF and BV values is not yet known. It is not clear from the 6 patients studied here if the unprocessed or motion-corrected pCT-derived parameter values are the most accurate reflection of tumour perfusion.

The only statistically significant difference between the unprocessed and motion-corrected parameter values was the reduction in BF at the tumour periphery in the motion-corrected images. Most of the tumour motion is seen at the edge of the tumour, so it is perhaps unsurprising that a change was seen here as a result of motion correction. The reduction of the BF value from the tumour periphery for one outlier (Patient D, baseline imaging) can explain this difference. Noise due to motion artefact at the edge of the tumour may have artificially elevated this patient's BF measurement on the pre-nelfinavir imaging prior to motion correction.

The test-retest variability in BF and BV for pancreatic tumours is not known. In a study of liver tumours that were analysed using the same software that has been used in this study, the scan-rescan variability of BF and BV were 11.2% and 14.4% respectively.²³² The method used here by Ng *et al.* was superior to the method employed here, as 4D perfusion imaging

was performed. If we were to apply this level of uncertainty to the change in BF and BV observed in this study, even if it could be considered a conservative estimate of the uncertainty in our parameter values, only 2 of the 5 patients would have had a significant change in BF and BV as a result of nelfinavir therapy when there was correction for tumour motion.²³² A greater understanding of the uncertainty of the parameters in the setting of LAPC is essential before drawing clear conclusions from changes in these parameter values.

3.4.5 Moving towards the personalisation of radiotherapy dose prescriptions in LAPC using functional imaging

While an increase in radiotherapy dose has been shown to impact upon survival in LAPC,²³³ this is at the expense of increased treatment-associated toxicity.^{234,235} Targeting a subvolume within the GTV may allow an increase in dose without compromising normal tissue constraints, particularly if 4D imaging was used to allow for a personalised, reduced margin.

Of the 17 patients who had paired FDG-PET scans analysed here, 4 patients had a mCR, so increasing the dose to the FDG-avid area would not have improved the imaging outcome. Care should be taken to avoid regarding mCR as 'cure'. Residual FDG uptake was taken as an end-point in this study as it was thought to equate to a radioresistant subvolume within the tumour. The aim of any further dose escalation should not just be the establishment of mCR, but rather pathological complete response and long-term tumour control. While a fall in SUV_{max} greater than the median value in a cohort of patients was found to be predictive of an improved OS in the ARC 2 study (see Chapter 2) and OS and PFS in a published series,²³⁶ another study required inclusion of a baseline SUV_{max} measurement (< 3.5) or a decrease in SUV_{max} of $\geq 60\%$ from pre- to post CRT to accurately identify those with

favourable OS and PFS.⁴⁴ To date, no large-scale trials have definitively demonstrated that a mCR equates to an overall survival advantage in LAPC.

There are no published data to suggest that patients who achieve mCR will not derive benefit from an increased radiotherapy dose. Four patients in the FDG-PET series presented here did achieve mCR and it may be that they would not derive any benefit from dose escalation. This reinforces our belief that any future dose escalation studies should be isotoxic and that further evaluation of the impact metabolic response has on the prognostic utility of FDG-PET in LAPC is required. Selecting a smaller BTV, may make isotoxic dose escalation more easily deliverable than boosting the entire GTV.

The findings in Chapter 2 were that a pre-CRT FDG-PET $SUV_{max} < 6.2$ identified patients who were more likely to only ever have cancer localised to the pancreas. These are the patients who, intuitively, have the most to gain from targeted radiotherapy dose escalation. They are also the patients in whom generating a BTV based on 40% of the SUV_{max} may be the most challenging as 40% of a low SUV_{max} may lead to the inclusion of normal tissues in the boost volume. This further highlights the need for the clear definition of a tumour ROI for FDG-uptake quantification. It could be that FDG-PET is more useful in guiding treatment strategy selection than in BTV definition. One could also argue that those with higher pre-CRT FDG uptake who go on to develop MD do so because the radiotherapy dose was inadequate in establishing lasting local control, which allows further progression of the tumour, including at distant sites. If this hypothesis were to be true, FDG-PET may still have a role in BTV definition.

The best method of identifying which patients would most benefit from an intensification of therapy, accepting the inherent increase in treatment-associated morbidity, remains

unclear. Patients with intact Smad4 (Dpc4) expression tend to have a local pattern of progression compared with those with Smad4 (Dpc4) loss, who tend to have a distant pattern of progression.²¹ This suggests that tumours with intact Smad4 may benefit from an increased radiotherapy dose. While we have demonstrated that areas of residual FDG avidity can be identified from pre-treatment imaging, FDG-PET alone may not be the most robust way of risk-stratifying patients. It is likely that a combination of imaging and biological markers will offer the greatest discriminatory value. While the FDG-PET uptake volumes were spatially consistent in this study, this analysis was only possible in approximately 50% of the patients. An imaging modality that informs about a known cause of radioresistance that can be applied to all patients is required.

The relationship of hypoxia with chemo- and radioresistance is well documented. Delivering a higher radiotherapy dose to the region of the tumour that is known to be radioresistant is attractive. The use of 4 h FMISO-PET to derive a hypoxic BTV shows promise, as regions of peak FMISO uptake are geographically and quantitatively stable and the volumes of uptake are smaller than the GTV. The uncertainties in FMISO-PET-derived hypoxic BTVs need to be understood, and reduced, before radiotherapy dose painting based on these volumes is taken into a clinical setting.

Both the 4 h FMISO-PET, dynamic FMISO-PET and pCT imaging may be improved by using a 4D scanning protocol or reducing movement of the pancreatic tumour by using motion-mitigation techniques such as abdominal compression. Use of an abdominal compression device in one study reduced the mean craniocaudal movement of intra-abdominal tumours from 11.4 mm to 4.4 mm.²³⁷ This could reduce the uncertainty in FMISO-PET BTVs that were generated from time-averaged FMISO uptake during free breathing at 4 h.

Accounting for, or reducing, pancreatic tumour motion may reduce the noise in the dynamic FMISO-PET data, and therefore parameter maps, and pCT. This may allow for the coregistration of hypoxia and perfusion parameter maps to see how closely perfusion deficits and hypoxia spatially correlate in LAPC. If there is a close spatial correlation between areas of poor perfusion and FMISO uptake, it may be possible to use only one of these imaging modalities to derive a hypoxic BTV for radiotherapy dose escalation.

The predictive and prognostic utility of FMISO-PET in CRT for LAPC and how FMISO-PET parameters might be used in treatment strategy selection has not yet been investigated. The ARC2 study has now closed to recruitment and FMISO-PET scans were available in only 6 patients. A larger set of FMISO-PET images and patient treatment outcomes would be required for this type of analysis. A central hypothesis in this thesis is that it is not baseline tumour hypoxia that informs about patient outcome, but rather the failure of tumour reoxygenation during CRT. This has been observed in head and neck cancers where a failure of the tumour to reoxygenate by the end of the second week of CRT was associated with a poor treatment outcome.¹⁰³ Sequential FMISO-PET imaging during CRT for LAPC would therefore be needed to test this hypothesis in this patient group. A Phase II study (SCALOP2, NCT02024009) that randomises the use of nelfinavir concurrently with CRT or CRT alone in LAPC will open in 2016. Adding FMISO-PET imaging at baseline and towards the end of the second week of CRT would allow the effect of nelfinavir in improving tumour reoxygenation above that seen in standard CRT to be assessed. This cohort of patients is likely to be large enough to assess how FMISO-PET-defined hypoxia at baseline and during therapy relates to treatment outcome. The difficulties in applying a challenging imaging protocol across a multicentre trial will require some thought and planning.

3.5 Conclusions

Thresholding a pancreatic tumour using 40% of the SUV_{max} on baseline FDG-PET/CT identifies areas of residual metabolic activity seen on a post-CRT FDG-PET/CT. While the volume derived from 40% of the SUV_{max} predicts *a priori* the geographical location of residual metabolically active tumour post CRT in most patients, this analysis was only possible in approximately 50% of the patients. While the null hypothesis that there is no spatial relationship between areas of tumour FDG-uptake pre-CRT with areas of residual FDG-avidity post CRT can be rejected, this result needs to be interpreted in the clinical and population context. Any future dose-escalation studies that use this subvolume should be isotoxic to avoid increasing treatment-associated toxicity in those patients who may not derive therapeutic benefit.

FMISO uptake could be demonstrated in all 6 of the pancreatic tumours at both of the imaging time-points. Regions of peak FMISO uptake are spatially consistent which would support the use of FMISO-PET to define hypoxic BTVs for radiotherapy dose escalation and the null hypothesis that there is no spatial relationship between areas of FMISO uptake within pancreatic tumours on repeat testing can be rejected on the data presented here. While there was no change in maximum and peak FMISO uptake on the 4 h FMISO-PET from baseline to post-nelfinavir monotherapy, some evidence of a change was observed in FMISO retention kinetics in 4 out of 6 patients. This may suggest that 6–7 days of nelfinavir is able to modulate some hypoxic regions in the tumour, but a reduction in the regions of most intense hypoxia is not seen. These early results suggest that changes in blood flow could be observed as result of nelfinavir therapy as an improvement in BF was seen in the same patients who demonstrated a reduction in FMISO retention. A greater understanding of how to minimise uncertainty in pCT-derived perfusion parameters is required. Further

hypoxia and perfusion data pre- and post nelfinavir is required before the null hypotheses that nelfinavir has no effect on tumour hypoxia or perfusion can be rejected, though there is a suggestion that a hypoxia modulation as a result of increased perfusion may be seen in a proportion of patients.

Chapter 4: Predicting response to chemoradiotherapy in locally advanced rectal cancer – investigating a texture-based approach

4.1 Introduction

The utility of conventional anatomical imaging, such as CT- or MRI-derived changes, in differentiating those with pCR following CRT for LARC is uncertain. CT alone is poor at differentiating normal bowel wall post CRT from residual tumour.²³⁸ While some series have failed to show a correlation between MRI-volumetric change with rates of pCR³⁹ or TRG,⁴⁰ a qualitative assessment of post-CRT MRI appears to identify those who have failed to respond with a degree of accuracy, but performs poorly in predicting a good pathological response.⁴¹ While the focus of the work in this thesis is the application of functional imaging to the personalisation of CRT treatment, including improving response assessment, an improvement of the ability of routinely applied anatomical imaging modalities to accurately recognise pCR would help direct treatment decisions post CRT if an organ-sparing approach was to be considered without increasing the imaging burden to patients and with low additional cost.

Tumour biology is heterogenous within tumours, between tumours and over time. This heterogeneity can be the result of variations in BF, hypoxia, necrosis, non-cellular accumulations (e.g. mucin), cellular metabolism, proliferation and changes in cell surface receptor expression. Variation in pixel intensity and patterns of pixel intensity seen within a grey-scale clinical image of tumours can be quantified by a set of matrices and corresponding derived indices. This extraction of information on intratumoural heterogeneity from images using mathematical modelling is commonly referred to as 'texture analysis'. The term 'radiomics' is also being increasingly used to describe a method

of quantifying tumour imaging phenotype using large data sets that characterise tumour texture, shape and pixel intensities.²³⁹⁻²⁴¹

Certain texture features of clinical CT images have been correlated with patient outcomes. Texture-based biomarkers from CT images have been associated with overall OS in HNSCC¹⁹⁴ as well as NSCLC.¹⁹⁵ CT-derived texture features have also successfully predicted the incidence of early tumour recurrence following stereotactic ablative radiotherapy¹⁹⁶ and response to chemotherapy²⁴² in NSCLC.

A wide range of radiomic features, and how they may be derived from images, have been described. Up to now, most studies of the texture analysis of images in oncology have used in-house software using a limited number of radiomic parameters. There is a need for a publicly available software tool that allows for the derivation of a comprehensive panel of radiomic features.

Previous studies have identified that the large number of statistical tests applied to the large number of parameters derived from texture analysis reduces confidence in the statistical significance of any observed difference in the parameter between groups. A principal component analysis (PCA) has therefore been recommended as it reduces the dimensionality of the large data set derived from a texture-based approach²⁴³ to allow for recognition of a radiomic 'signature' that can then be correlated with treatment response or patient outcomes.

It was hypothesised that variations in textural heterogeneity in rectal tumours may reflect differences in biological heterogeneity and that a number of these features, or a signature

of textural features, may be associated with response to CRT. The aims of the work presented in this chapter are therefore:

- To develop a radiomics software tool that allows for the comprehensive characterisation of quantitative features contained within grey-scale images.

The first step was to generate a comprehensive list of texture parameters that may be of interest. Dr Jun Li (Professor Mike Partridge's group) then developed the software to derive the radiomic parameters using standard equations outlined in the available literature for a panel of 62 texture features.

- To look for statistical associations between texture-based features and response to CRT. A secondary question of whether pre-CRT FDG-PET SUV_{max} improves the predictive function of the texture-based approach was also tested.

The ability of texture parameters derived from a pre-CRT, post-CRT and relative change in these texture parameters to predict tumour response was assessed. Pre-CRT FDG-PET SUV_{max} was added to the texture parameters to see if this improves the predictive power of the image-derived signature. Tumour response was defined in two ways – pCR (Mandard TRG 1 only)⁵² versus non-complete response (Mandard TRG 2–5) and TRG 1–2 versus TRG 3–5. Prediction of Mandard TRG 1–2 has been the focus of many functional imaging studies. Patients with Mandard TRG 1–2 have a better prognosis than those with TRG 3–5 in terms of local failure, metastases-free survival and OS.⁵⁸ If pCR cannot be predicted using a texture-based approach, accurately identifying Mandard TRG 1–2 may be a good first step towards using a radiomics approach to guide individual treatment strategy selection.

The null hypothesis to be tested states that there is no association between CECT texture features and response to CRT in LARC.

4.2 Methods

4.2.1 Texture parameter identification and software development

To find the most commonly used parameters used in the textural analysis of cancer imaging, a literature search was performed. Studies were identified using both Ovid MEDLINE® and In-Process & Other Non-Indexed Citations and Ovid MEDLINE® 1946 to present and Scopus, using the search terms 'Texture analysis or Radiomics' and 'Cancer' and 'Response prediction'. A list of texture parameters was generated from these studies and the references to the underlying method of the texture analysis were obtained. Dr Jun Li then developed a software tool that applies each image analysis method to grey-scale images using MatLab (version 2013a, MathWorks, Natick, MA, USA). The software tool (Texture Analysis Toolbox) then exports the derived texture parameter values for the analysed image into Microsoft Excel. The Texture Analysis Toolbox also describes the 3D shape of any ROI and provides summary statistics of the pixel intensity histogram.²⁴⁴

4.2.2 Texture Analysis Toolbox and parameter extraction

The toolbox was able to generate 62 texture parameter values from all of the CECTs. The parameters fall into three categories: 3D morphological, 3D non-spatial and spatial textural characteristics. The 3D features were generated from the delineated tumour ROI as they concern tumour shape or signal intensity. The 3D morphological variables describe the shape and volume of the tumour. The parameters quantify voxel number, TV, length, surface area, sphericity, cylindricity, effective spherical radius and effective cylindrical radius.

The 3D non-spatial characteristics are concerned with pixel values and their distribution characteristics. 2D spatial textural characteristics were derived from the following texture matrices: grey-level co-occurrence matrix, grey-level run-length matrix, grey-level size-zone matrix, neighbouring grey-level dependence matrix and neighbouring grey-tone dependence matrix. The spatial textural characteristics were calculated from a bounding box from around the tumour ROI on each axial section. The 2D values were then converted to a weighted mean to generate a 3D parameter value.

First-order (one pixel) parameters describe global textural features that relate to the grey-level frequency distribution of the image,²⁴⁵ while second-order (two pixels) are based on joint conditional probability density function within the ROI.^{246,247} In grey-level co-occurrence matrix, the parameter quantifies the number of times that defined intensity levels occur in two pixels separated by a set distance in a defined direction. Grey-level run length counts the number of pixel segments having the same intensity in a given direction (e.g. 0°, 45°, 90°, or 135°) in an image, which results in a matrix in which the parameter describes a grey level and the number of segments of a defined length.²⁴⁷⁻²⁴⁹ A run is a string of consecutive pixels that have the same grey-level intensity along a direction.²⁴⁹ The neighbouring grey-level dependence matrix records the number of pixels having defined grey levels as neighbouring pixels within a defined distance.²⁵⁰ The parameters defined by the neighbourhood grey-tone difference matrix is a summation of the differences between the grey level of all pixels with the defined grey level and the average grey level of their surrounding neighbours.²⁵¹ A description of all of the texture, but not volumetric, parameters that are quantified using the texture analysis software can be seen in Table 18.

A description of the governing equations of each parameter and the literature source for each descriptor can be found in Appendix B.

Table 18: A description of the texture features that are quantified by the Texture Analysis Toolbox.

	Parameter	Description
First-order statistic	m1	Mean of normalised grey level
	v1	Variance of grey levels
	sd1	Standard deviation of grey levels
	s1	Disorder or uncertainty of pixels described by grey levels
	cov1	Standardised measure of dispersion of ROI pixel distribution
	max1	Maximum value of pixels in ROI (normalised)
	min1	Minimum value of pixels in ROI (normalised)
	vol1	Number of pixels in ROI
	diax	Diameter of the ROI/lesion, x direction
	diay	Diameter of the ROI/lesion, y direction
	skew	Measures the asymmetry of the pixel distribution in ROI about its mean
	kurt	Measures the 'tailedness' or outliers of the pixel distribution in ROI
	m2	Mean of grey level
	cov2	Measure of dispersion of ROI pixel distribution
	max2	Maximum value of pixels in ROI (original)
	min2	Minimum value of pixels in ROI (original)
Grey-level co-occurrence matrix	HM	Measures the uniformity of the non-zero entries in the matrix with a range of [0, 1]. A high homogeneity means an image has little variation
	EG	A measure of local homogeneity. The higher the energy value, the bigger the homogeneity of the texture
	CV	Measures how much two grey levels change together
	CR	The normalised coefficient of variance
	CT	Refers to a local grey-level variation. High contrast values are expected for heavy textures and low for smooth, soft textures
	ET	A measure of image spatial disorder. A completely random distribution has high entropy
	DS	Similar to CT, by a weight difference
Grey-level run-length matrix or Grey-level size-zone matrix	SRE	Measures the distribution of short runs and is expected to be large for fine textures. (A run is defined as a string of consecutive pixels which have the same grey level intensity along a specific linear orientation)
	LRE	Measures the distribution of long runs and is expected to be large for coarse structural textures
	GLN	Measures the similarity of grey level values. The value will be small if the grey level values are alike
	RLN	Measures the similarity of the length of runs. The value is expected to be small if the run lengths are alike
	RP	Measures the homogeneity (and the distribution) of grey levels
	LGRE	Measures the distribution of low grey level values. The value is large for an image with low grey level values

	HGRE	Measures the distribution of high grey-level values. The value is large for an image with high grey-level values
	SRLGE	Measures the joint distribution of short runs and low grey-level values. The value is large for an image with short runs and low grey-level values
	SRHGE	The value is large for an image with short runs and high grey-level values
	LRLGE	The value is large for an image with long runs and low grey-level values
	LRHGE	The value is large for an image with long runs and high grey-level values
Neighbouring grey-level dependence matrix	SNE	Measures the distribution of small number of neighbours and is large for fine textures
	LNE	Measures the distribution of large number of neighbours and is large for coarse textures
	NNU	Measures the similarity of numbers of neighbours. The value is expected small if the numbers of neighbours are alike
	SCM	Second moment measure of the fluctuation of numbers of neighbours from its mean
	ENT	Measures the disorder of numbers of neighbours. A random distribution would have very high entropy
Neighbourhood grey-tone difference matrix	COA	Coarseness shows the level of spatial rate of change in intensity. Large values represent that grey-tone differences or the probability of occurrence are small, i.e. coarse texture
	CON	Contrast examines different intensity levels. High contrast means that the intensity difference between neighbouring is large
	BSY	Busyness shows intensity changes from one pixel to its neighbour. A small change means a high level of local uniformity in intensity
	COM	Complexity – a texture is considered complex if the information content such as patches or primitives (e.g. edges, lines) is high. COM has an inverse relationship with the sizes of primitives and/or probabilities of occurrence of grey-tone values. High COM indicates a high degree of information content
	TST	Texture strength (TST) is high if the primitives are easily definable and clearly visible. Hence a high value of TST corresponds to a strong texture

4.2.3 Patient selection

Patients were identified from a cohort recruited to an ethically approved imaging study (Regional Ethics Committee Reference number: 06/Q1604/162) of LARC with preoperative, downstaging CRT. Patient records were examined and patients with documented biopsy-proven rectal adenocarcinoma, adequate staging information in the patient record, CECT scans both pre- and 6 weeks post CRT that were available for review and documentation of Mandard TRG by a consultant histopathologist were eligible for inclusion in this analysis.

4.2.4 Chemoradiotherapy regimens

All patients were treated using standard, departmental CRT schedules. Patients were recruited between 2007 and 2012 over which time two different treatment strategies were delivered. All patients received either 45 Gy in 25 fractions concurrently with 5-fluorouracil (5-FU) 4000 mg/m² over 96 h starting on day 1 and day 29 or 50.4 Gy in 28 fractions concurrently with capecitabine 900 mg/m² for 5 days a week of the whole treatment course. All radiotherapy was delivered using a three-field, 3D-conformal technique delivering 1.8 Gy per day, 5 days a week to a planning target volume that included the tumour, mesorectum and pelvic lymph nodes.

4.2.5 Imaging protocols

To replicate routine clinical practice, any available CECT at the appropriate time-points were used for texture analysis. Where possible, the contrast-enhanced radiotherapy planning CT was used for the pre-CRT image analysis to reduce the heterogeneity of imaging protocols used. For all other scans, including the post-CRT imaging, details of the CT imaging protocol were obtained from the image DICOM header file. The scanning protocol used to acquire the images was recorded so that variation in CT acquisition protocols could be factored in to any future statistical analyses that identified a predictive function of a radiomic signature.

All FDG-PET imaging was performed locally using the same protocol as described in Chapter 2. In short, scans were performed on a GE Discovery 690 (GE Healthcare, Buckinghamshire, UK) after fasting for 6 h and ensuring that the blood glucose was <10 mmol/L; 4 MBq/kg (up to 600 MBq) of FDG was injected and a 10 min acquisition was started after an uptake time of 90 min.

4.2.6 Defining a region of interest for texture analysis

All ROI outlining was performed using Eclipse software (version 13.0, Varian Medical Systems, Palo Alto, CA, USA). The tumour was outlined on the pre-CRT CECT. Where possible, a contemporaneous T2-weighted MRI was rigidly coregistered to the CECT to aid accurate delineation. A ROI labelled 'tumour' was generated. The pre-CRT CECT was then rigidly registered to the post-CRT CECT prioritising accurate soft tissue matching in the rectum. After visual inspection of the registration, the tumour ROI was propagated across to the post-CRT CECT. Again, where possible, a post-CRT T2-weighted MRI was rigidly registered to the post-CRT CECT. The tumour ROI was then edited to match the resultant TV. When no residual tumour could be seen on the post-CRT scan, the rectal wall at the site of the original tumour was outlined. An example of a patient with no residual TV and how the tumour and rectal wall was outlined can be seen in Figure 38.

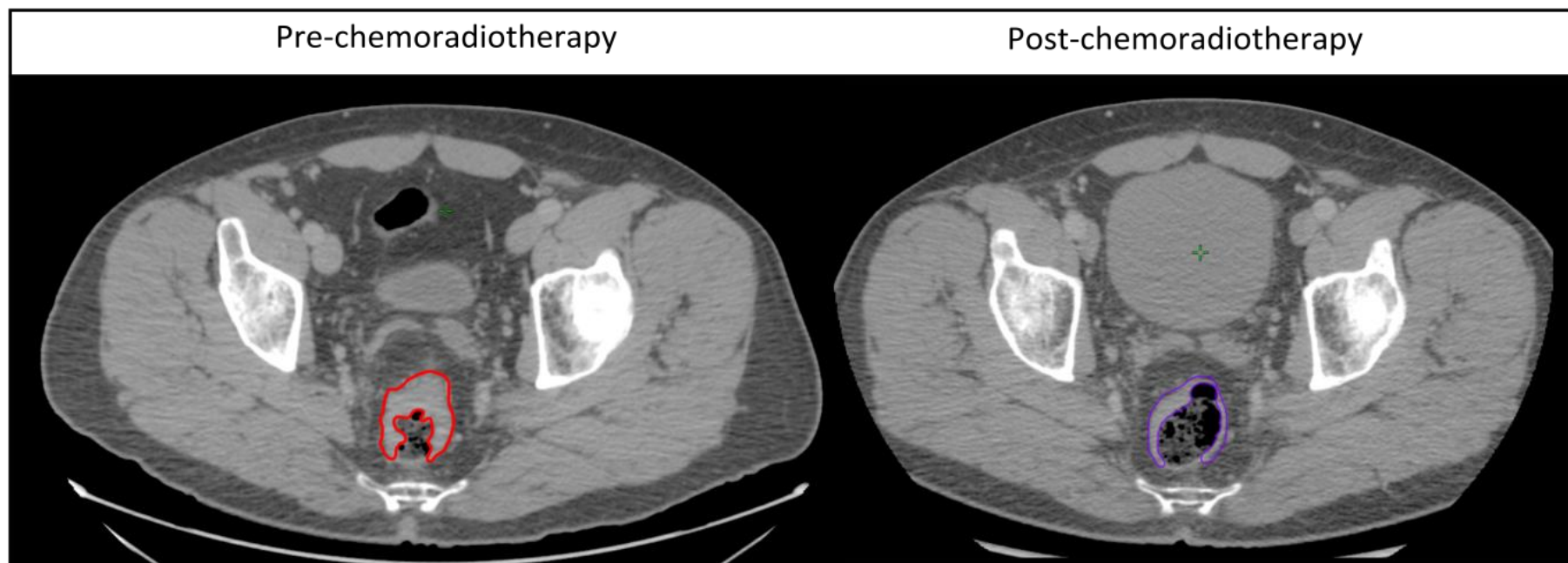


Figure 38: Defining a ROI for texture analysis of a rectal tumour on contrast-enhanced CT. The pre-CRT TV (red) had to be modified on the post-treatment CECT (purple). Histological assessment of the resected rectum in this patient revealed a pathological complete response.

4.2.7 Image analysis

4.2.7.1 Texture analysis of contrast-enhanced CT images

The CECT images and ROIs were analysed using Dr Jun Li's Texture Analysis Toolbox in MatLab (Toolbox). The toolbox mapped all of the pixel grey-levels in the images (with CT Hounsfield units (HU) from -160 HU to 240 HU) to grey levels 0 to 255 respectively (256 grey level 'bins'). The texture matrices were then generated. Parameters were then derived from these matrices on each 2D axial image. A weighted mean of these parameters was taken. Texture parameters derived from these matrices were calculated from a rectangle of closest fit around the tumour ROI to preserve texture information from the edge of the tumour region. Parameters that are derived from the pixel intensity histogram were obtained from pixels contained within the ROI plus a one-pixel margin around the ROI. 3D morphological characteristics, shape features, such as surface area, sphericity and cylindricity were calculated from the tumour ROI only.

4.2.7.2 FDG-PET SUV_{max} calculation

The attenuation corrected FDG-PET/CT image was viewed in Hermes software (version 2.2B, Hermes Medical Ltd, London, UK). A ROI was drawn around the area of uptake at the site of the rectal tumour and the SUV_{max} obtained.

4.2.8 Statistical methods

The statistical analysis of the image-derived parameters was performed in collaboration with Dr Christiana Kartsonaki. All tests were performed in R statistical software (www.r-project.org). PCA and hierarchical clustering was performed on the texture data with the addition of SUV_{max} for each patient at each time-point. The PCA and hierarchical plots were visually examined to see if there was any difference in texture and SUV_{max} patterns

between patients with different TRGs. Heat maps were generated from the hierarchical clusters to visualise any potential clustering of texture features with TRG. Logistic regression was then used one variable at a time to fit a model for predicting pCR versus non-pCR and then again to fit a model to predict either Mandard TRG 1–2 or Mandard TRG 3–5. The variables with promising p values were chosen using a multivariate logistic regression model.

4.3 Results

4.3.1 Patient characteristics, chemoradiotherapy regimens and Mandard tumour regression grade

The clinical features and Mandard TRG of patients included in the image analysis can be seen in Table 19. Twenty-six patients were included in the study. The median age of the patients was 66 years (range 46–80). Only 4 (15.4%) patients achieved pCR (Mandard TRG 1) with 20 (76.9%) patients having a ‘good’ response (Mandard TRG 1–2). Six (23.1%) patients had a ‘poor’ response to CRT (Mandard TRG 3–5) although it should be noted that all 6 of these patients had Mandard TRG 3. No patients had Mandard TRG 4 or 5.

Table 19: Patient characteristics, CRT schedule and Mandard TRG.

	n (%)
Total no. of patients	26 (100.0)
Male	20 (76.9)
<i>T stage</i>	
3	21 (80.8)
4	5 (19.2)
<i>N stage</i>	
0	8 (30.8)
1	7 (26.9)
2	11 (42.3)
<i>Chemoradiotherapy schedule</i>	
5-FU + 45 Gy*	6 (23.1)
Capecitabine + 50.4 Gy*	20 (76.9)
<i>Mandard TRG</i>	
1	4 (15.4)
2	16 (61.5)
3	6 (23.1)
4	0 (0.0)
5	0 (0.0)

*All radiotherapy given in 1.8 Gy per fraction: 45 Gy in 25 fractions, 50.4 Gy in 28 fractions

4.3.2 Heterogeneity of CT scanning protocols

Ten different CECT scanning protocols were used to acquire the 52 CECT images used in this analysis. A combination of both GE and Siemens scanners were used to acquire the images, and the volume and type of the contrast agent used varied between protocols. The slice thickness of the CT was 2.5 mm in 37 of the scans and 5.0 mm in 15 of the CT scans.

4.3.3 The predictive utility of CT texture parameters in locally advanced rectal cancer

4.3.3.1 No difference was seen in the texture features of patients with different responses to chemoradiotherapy

PCA plots for textural features tumours seen on CECT pre- and post CRT and the difference in parameters from pre- to post CRT and how they relate to Mandard TRG can be seen in Figures 39 to 41. No clear patterns in the PCA from pre- and post CRT and relative change in the texture parameters and how they relate to Mandard TRG were seen.

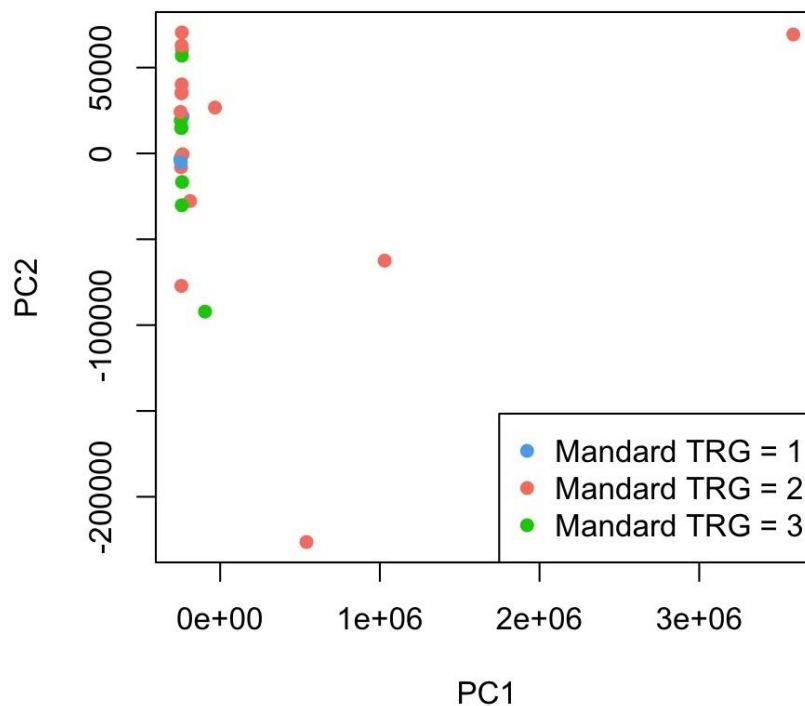


Figure 39: PCA for texture features of patients with differing responses to CRT as described by Mandard TRG. Texture features were extracted from a pre-CRT CECT.

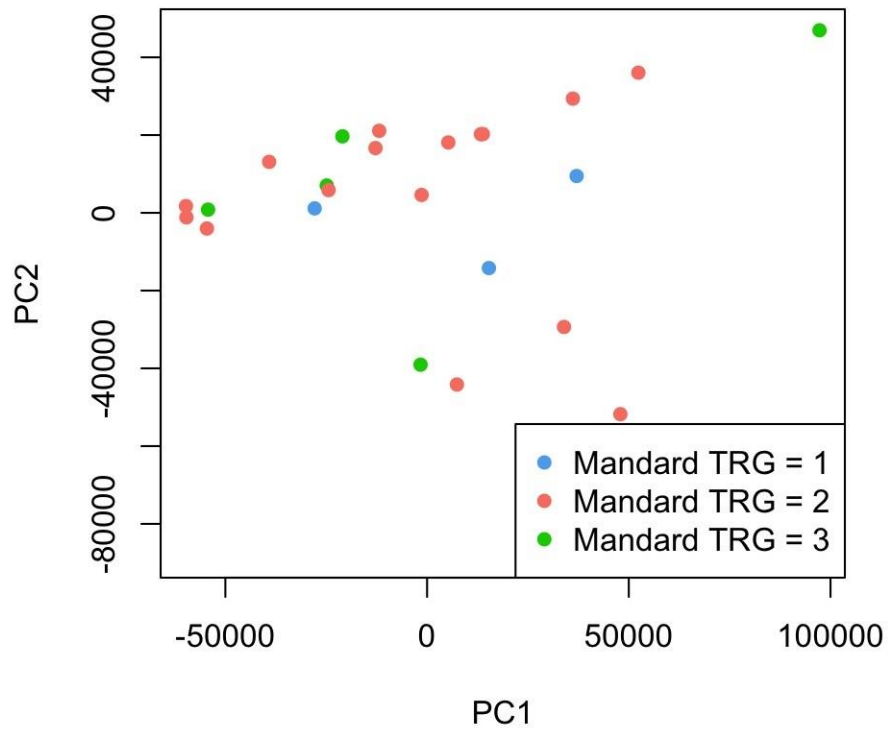


Figure 40: PCA for texture features of patients with differing responses to CRT as described by Mandard TRG. Texture features were extracted from a post-CRT CECT.

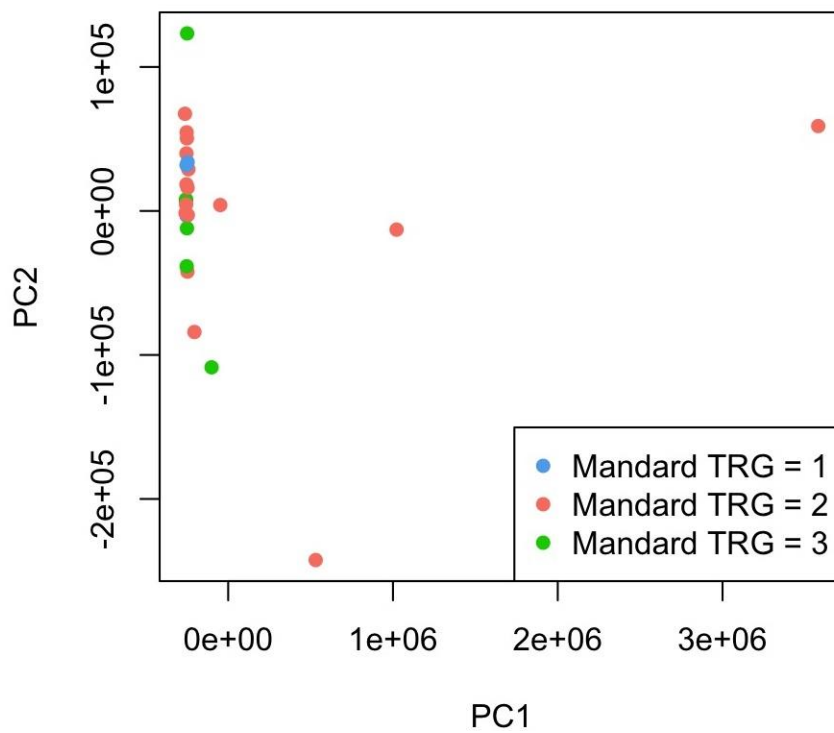


Figure 41: PCA for the difference in texture features from pre- and post-CRT CECTs of patients with differing responses to CRT as described by Mandard TRG.

The hierarchical cluster plots and heat maps for texture parameters derived from pre- and post-CRT CECTs and the relative difference in texture-based parameters can be seen in Figures 42 to 44. It can be seen that there was no characteristic clustering of texture features from pre- or post CRT, or in the relative change in the texture features in patients with the same response to treatment as described by Mandard TRG.

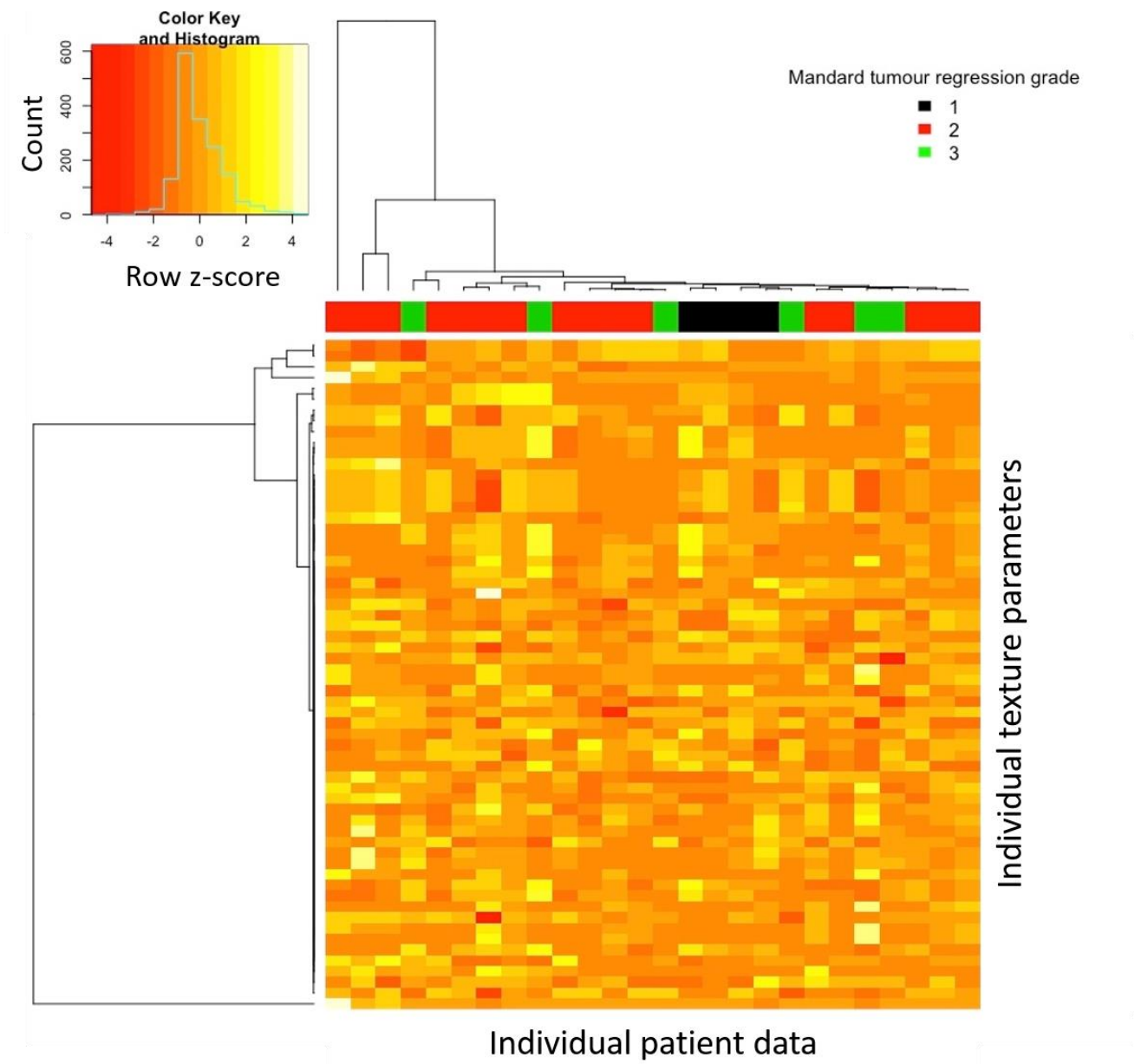


Figure 42: Hierarchical clustering and heat maps of texture features in relation to response to CRT (Mandard TRG) based on texture features from a pre-CRT CECT.

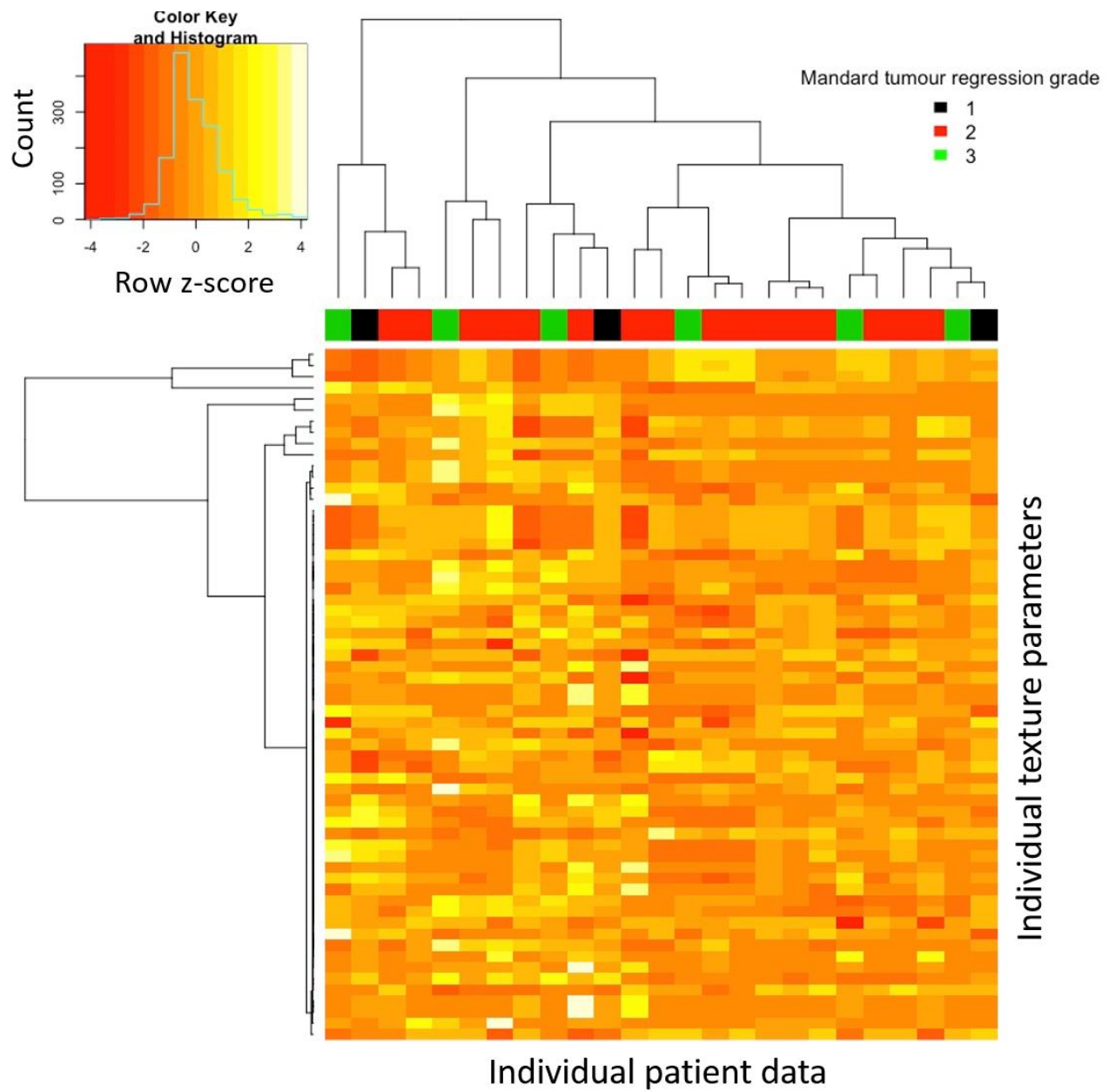


Figure 43: Hierarchical clustering and heat maps of texture features in relation to response to CRT (Mandard TRG) based on texture features from a post-CRT CECT.

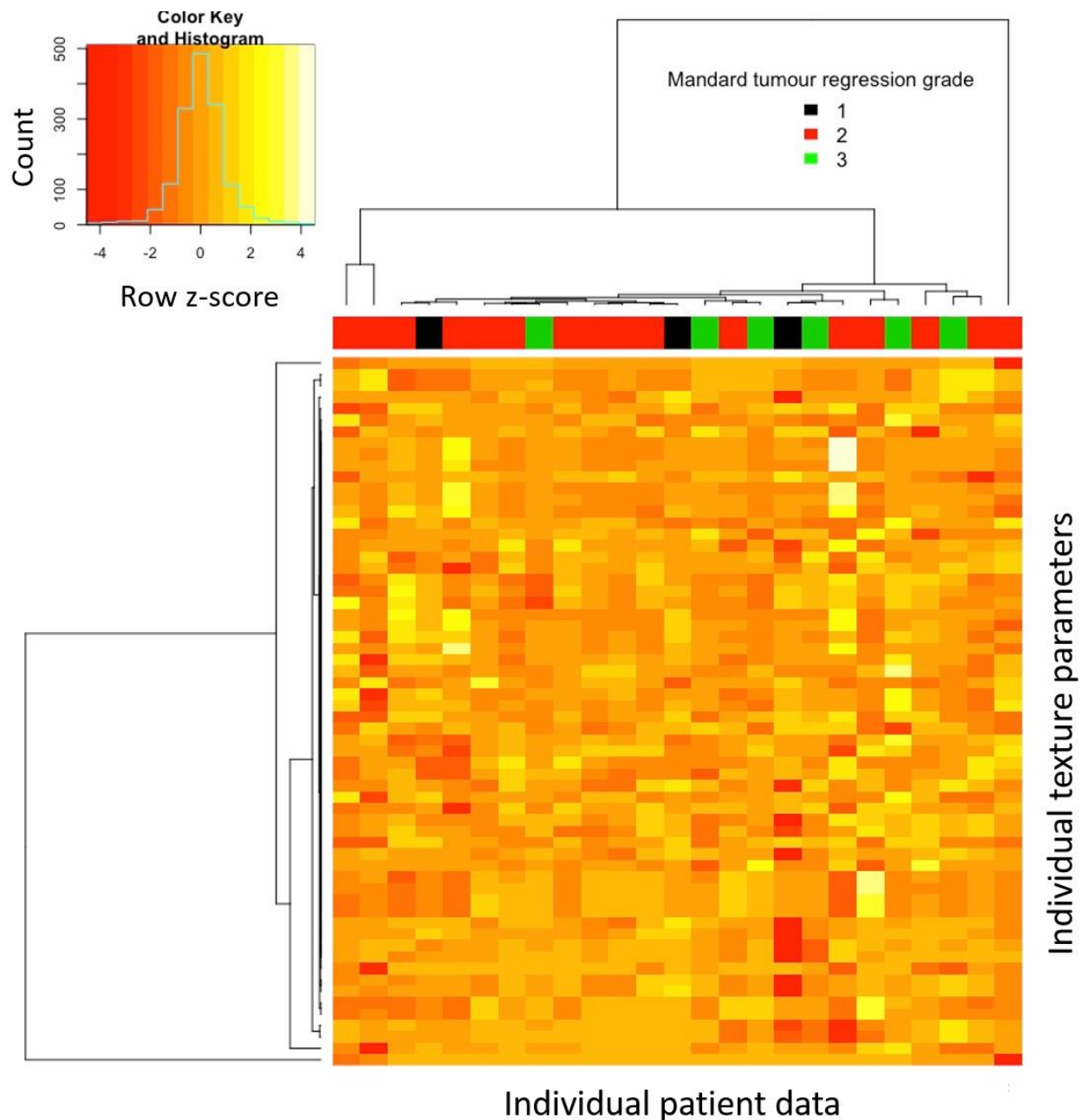


Figure 44: Hierarchical clustering and heat maps of texture features in relation to response to CRT (Mandard TRG) based on the change in texture features from CECTs obtained pre- and post-CRT.

4.3.3.2 *Texture analysis of pre-chemoradiotherapy CECT does not predict pathological complete response*

None of the texture variables was significantly associated with the pCR. The variables with a p value ≤ 0.2 were chosen to be fitted together in a logistic regression model. These were: grey-level co-occurrence matrix parameter 'entropy', ROI surface area, grey-level size zone

matrix run percentage, ROI length, ROI effective spherical radius, grey-level run-length matrix, low grey-level run emphasis, run percentage and ROI volume. However, the fitting algorithm did not converge, so a model with the entropy, ROI surface area and run percentage which had given a p value ≤ 0.1 was fitted instead. None of these was significantly associated with outcome.

4.3.3.3 Pre-CRT FDG-PET SUV_{max} does not improve the utility of pre-CRT image analysis in prediction pathological complete response

On univariate analysis, pre-CRT SUV_{max} did not correlate with pCR (p = 0.485). Addition of SUV_{max} considerably affected the predictive model insofar as different texture parameters were then suggested for the multivariate model. A model with all variables with a p value ≤ 0.25 (ROI sphericity, low grey-level run emphasis and entropy) and SUV_{max} was fitted, but no significant association was found.

4.3.3.4 Texture analysis of post-CRT CECT does not predict for pCR with or without the inclusion of FDG-PET SUV_{max}

A model with all variables with a p value ≤ 0.2 (grey-level co-occurrence matrix entropy, ROI sphericity, grey-level run-length matrix short run high grey-level emphasis, ROI surface area, short run emphasis, low grey-level run emphasis, ROI length and ROI cylindricity and grey-level size zone matrix run percentage) was fitted. The fitting procedure did not converge. Then a model with all variables with a p value ≤ 0.1 (entropy, ROI surface area and run percentage) was fitted. No significant association was found. A model with all the variables with p value ≤ 0.2 in addition to pre-CRT SUV_{max} was fitted, but it did not converge. Variables were removed one at a time but the fitting algorithm did not converge for any of the models.

4.3.3.5 The relative change in texture parameters did not predict pCR. Addition of pre-CRT

FDG-PET SUV_{max} did not identify a predictive model

The relative change in texture variables that had a p value ≤ 0.2 on univariate analysis were chosen to be fitted together in a logistic regression model. These were: ROI volume, coefficient of variation, voxel filled number, ROI variance, run percentage, ROI effective spherical radius, ROI standard deviation, low grey-level run emphasis, ROI length, ROI entropy, short run emphasis, short run high grey-level emphasis, run length non-uniformity. However, the fitting algorithm did not converge, so variables were removed one at a time until a model with ROI volume, coefficient of variation and voxel filled number was fitted. None of these was significantly associated with outcome.

A multivariate model with all variables with a p value ≤ 0.15 (run length non-uniformity, coarseness, run length non-uniformity, ROI cylindricity, short run emphasis, coefficient of variation, variance, standard deviation, run percentage) and SUV_{max} was fitted, but it did not converge.

4.3.3.6 Pre- and post-CRT CECT texture features and relative change in texture feature

with or without pre-CRT FDG-PET SUV_{max} does not predict Mandard TRG 1–2

An identical process to the one described above was carried out to attempt to predict Mandard TRG 1–2 rather than pCR (TRG 1). None of the variables was significantly associated with TRG 1–2 on univariate analysis. None of the multivariate models to predict Mandard TRG 1–2 derived from pre- or post-CRT parameters or the relative change in parameters was seen to converge.

4.4 Discussion

CT imaging at diagnosis and following downstaging CRT is routinely performed in patients with LARC. A radiomics approach to quantifying tumour heterogeneity in CT images has previously identified prognostic and predictive radiomic ‘signatures’ in a number of tumour types,^{243,252} but this approach has not yet been described in rectal cancer. Personalisation of cancer treatment strategies is a current research priority. Clinical practice is moving in the direction of using molecular targeting therapy and trials, such as the currently recruiting FOCUS4 trial (EudraCT number 2012-005111-12) in metastatic colorectal cancer, to stratify treatment selection based on molecular profiling. Texture analysis, or a radiomics-based approach, has accurately predicted O⁶-methylguanine-DNA-methyltransferase mutation in glioblastoma multiforme,²⁵³ codeletion of 1p and 19q, a marker of good prognosis, in oligodendroglioma²⁵⁴ and p53 mutation in HNSCC.²⁵⁵ A significant correlation between radiomic features and gene expression patterns has been observed in NSCLC, with the additional observation that increasing intratumoural heterogeneity is associated with cell cycle pathways suggesting an increase in proliferation in the more heterogeneous tumours.²⁵²

Identification of an imaging biomarker, or set of biomarkers, that can inform about tumour biology, including predicting response to CRT, would provide a non-invasive, cost-effective method of personalising treatment strategy decisions for patients with LARC. Unfortunately, radiomic features of the rectal tumours analysed in this study did not accurately predict response to CRT – even with the addition of the pre-CRT FDG-PET SUV_{max}. The role of a radiomics-based approach to inform of molecular heterogeneity warrants further investigation and may identify patients who may benefit from the combination of molecular targeting agent with standard CRT.

Only 4 out of 26 patients included in this analysis went on to have pCR post CRT and only 6 out of 26 had a 'poor' response – Mandard TRG 3. With such small numbers of responders and with no patients having minimal or absent response (Mandard TRG 4–5), it is perhaps unsurprising that a radiomic signature that predicted response was not identified. A larger cohort of patients with a wider range of CRT responses may help identification of an imaging signature to guide therapy decisions in LARC. As post-CRT MRI is useful in identifying patients who have failed to respond,²⁵⁶ the lack of patients with Mandard TRG 4 and 5 in this group of patients may reflect local practice to give consolidative chemotherapy to those who appear to have not responded to CRT.

The method of image analysis employed here needs to be refined before concluding that a radiomic approach to rectal cancer imaging analysis is not predictive of CRT response. A spatial grey-level matrix approach to the textural analysis of images was first presented by Haralick *et al.* in the 1973.²⁵⁷ A consensus on the optimal number of grey levels for co-occurrence matrix calculations has not yet been reached. It could be that the grey-level bin width needs to be optimised for each tumour cell type and could depend on the treatment outcome to be predicted. In this analysis, the default window from –160 HU to 240 HU was mapped to 256 grey-level bins. This predictive utility of texture parameters derived from the texture matrices may be improved by limiting the number of grey-level bins. Studies often limit the number of grey levels to 16, 32, 64, or 128.²⁵⁸⁻²⁶⁰ It could be that using too many grey levels has a detrimental effect on the texture quantification. For example, runs may only ever contain only one pixel. While the ability of texture parameters from contrast-enhanced MRI to predict treatment outcome in breast cancer did not appear to be altered by varying the number of grey-level bins,²⁴³ an understanding of how the

number of grey levels chosen impacts upon the derived parameter values from CT images in rectal cancer is lacking.

As well as a need to investigate the number of grey levels individual pixels are assigned to, the most appropriate pixel separation is not yet known. All pixels in the CT images here were quantified individually. It could be that clustering of pixels, with averaging of intensity values within the clusters, may generate texture patterns that more accurately reflect the underlying tumour biology.

The distance between axial sections of a CECT is considerably larger than the distance between pixels. It is therefore difficult to justify a fully 3D-based approach to texture analysis – particularly those derived from co-occurrence matrices. While the results presented here have derived texture statistics from a 3D TV, this has been done by taking weighted means across a series of 2D axial CT images. Other studies have also taken this approach of viewing a TV as a series of 2D images.²⁴⁹ Some authors suggest that a 3D approach is preferable to 2D^{261,262}, and in a large series of patients demonstrating a correlation of texture features with prognosis and gene expression in HNSCC and NSCLC, the texture features were calculated in all three dimensions.²⁵² Another approach has been to perform analysis on a single 2D axial image, but use functional imaging, such as the slice that contains FDG-PET SUV_{max}, to identify the most appropriate axial section to quantify texture features.²⁶³

In this series, texture parameter values were extracted from a manually delineated ROI with an additional one-pixel margin, an approach that has been employed in most series that have investigated the characterisation of medical images using radiomics.²⁵² Using a semi-automated means of tumour segmentation may produce more reproducible tumour

volumes and therefore more reproducible texture parameters. This may then allow the test-retest repeatability of texture parameters from CT images of rectal cancers to be characterised. A low baseline variability in radiomic features is required. Radiomic features derived from FDG-PET images using a manually defined ROI have been shown to have acceptable test-retest repeatability and inter-observer reproducibility in NSCLC.²⁵⁸ The test-retest repeatability of texture features of lung tumours was found to be acceptable even when the tumour ROI was segmented manually.²⁶⁰ When the test-retest repeatability of texture features of FDG-PET images in oesophageal cancer was tested, the baseline variation in texture parameters was found to be less than that observed in SUV based parameters.²⁵⁹ The test-retest repeatability of the Texture Analysis Toolbox with CT images of rectal cancer requires further investigation.

It should also be remembered that using different image sequences, even from the same imaging modality at the same time-point, results in completely different patterns of texture.²⁶⁴ Also, the value of the texture parameters varies considerably depending on when the image is taken in relation to contrast injection in MRI studies in breast cancer.²⁴³ It is routine practice to perform MRI imaging pre- and post CRT for LARC in the UK. If MRI was to be taken forward as the imaging modality of choice to try to predict CRT treatment outcome in LARC, it should be remembered that the imaging protocol and the sequence used for texture analysis leads to marked differences in the texture parameters.²⁶⁴ Ten different imaging protocols were used to acquire the CT images analysed in this study. In a large series of patients with NSCLC and HNSCC, variation in CT acquisition protocols did not affect the prognostic performance of the radiomic signature.²⁵² The authors propose that this supports that translational potential of a radiomics approach across multiple centres,

but they also speculate that better standardisation of imaging protocols may increase the power of radiomics even further. Application of the radiomic signature derived from NSCLC to HNSCC revealed that the signature performed better in the HNSCC cohort. It is thought that this is a result of the HNSCC images being acquired with patient immobilisation whereas the lung images would be subject to movement artefact. This further highlights the need for a standard, repeatable CT acquisition protocol that captures information on tumour texture with minimal image contamination from noise.

A systematic approach to the textural analysis of LARC is required if a radiomics quantification method is to be used as a predictive tool to guide treatment strategy selection. Each step of the proposed process in Figure 45 requires optimisation if a larger cohort of patients were to be recruited to a study that aimed to identify a radiomic signature that could be used to predict CRT response in LARC.

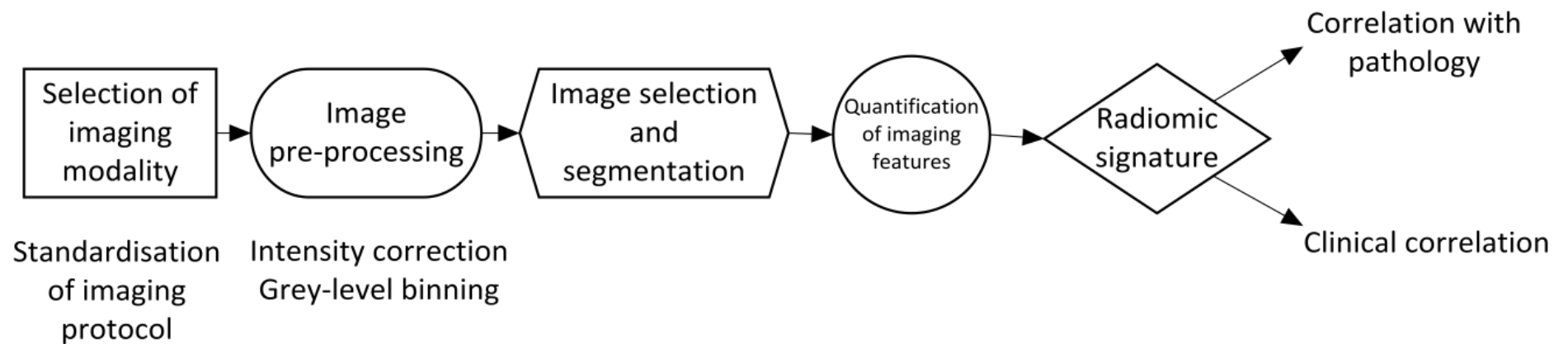


Figure 45: Steps that need to be optimised in order to assess the utility of a textural approach to rectal cancer imaging analysis in describing tumour biology and guiding treatment strategy selection.

4.5 Conclusion

We have successfully developed an image texture analysis tool that is able to derive all the currently published texture parameters. It has been made available to the public for use. A larger cohort of patients treated with CRT for LARC with a diverse range of clinical outcomes is required to adequately test the predictive utility of the radiomic features that are quantified with the Texture Analysis Toolbox. A consistent approach to CT image acquisition is required before deciding that the texture features are not predictive. A greater understanding of the test-retest repeatability of the texture parameters generated by the software tool is required. A next step would be to limit the number of grey levels in the image by limiting the width of the HU 'bins'. This approach, may produce a texture map that is more in keeping with the resolution and inherent noise in the CT image. The radiomic features extracted from this simplified image may identify a signature that is useful in predicting CRT response. The null hypothesis that there is no association between CECT texture features and response to CRT in LARC is therefore accepted, but should be seen in the context of the limitations of the study as outlined above.

Chapter 5: Characterising changes in tumour hypoxia and perfusion during fractionated chemoradiotherapy for locally advanced rectal cancer

5.1 Introduction

The aim of the work in this chapter is to develop a clinical study that will:

- Identify and compare FMISO-PET-derived hypoxic volumes, using a variety of uptake thresholds, against whole-mount histology of pimonidazole in staining across the whole TV.

The FMISO uptake value that accurately represents intratumoural hypoxia is not known. A means of validating imaging-defined hypoxia against a histological ground truth needs to be established. The null hypothesis to be tested states that there is no spatial or quantitative relationship between areas of FMISO uptake and areas of pimonidazole staining on IHC.

- Quantify the test-retest variability of FMISO-PET and pCT in rectal cancer.

Characterisation of the baseline variation in FMISO-PET- and pCT-derived parameters is essential before quantification of the change in FMISO uptake and pCT perfusion parameters can be used to accurately describe hypoxia and perfusion modulation during fractionated CRT. The null hypothesis to be tested states that there is no relationship between parameters derived from FMISO-PET or pCT on repeat testing.

- Assess the utility of FMISO-PET in deriving a hypoxic biological target volume that could be used for selective radiotherapy dose boosting.

The region of FMISO would need to be spatially consistent within the tumour during the course of CRT and should be smaller than the GTV. The null hypothesis to be tested states that areas of FMISO uptake seen on PET are not spatially or volumetrically consistent.

- Quantify changes in tumour hypoxia and perfusion during the first 2 weeks of CRT.

A hypothesis central to this thesis is that it is the failure of tumour reoxygenation informs about CRT outcome better than a pre-CRT assessment of hypoxia. Heterogeneity in rates of reoxygenation will be sought using FMISO-PET. To explore the dependence of reoxygenation on an increase in perfusion, tumour perfusion will be assessed using pCT and dce-MRI. The null hypothesis to be tested states that there is no change in tumour perfusion from pre-CRT to the end of the second week of CRT.

- Investigate the relationship between serum OPN levels and tumour FMISO uptake.

An understanding of how circulating OPN levels reflect tumour hypoxia may give confidence in using OPN to risk-stratify tumours based on their hypoxic status. The null hypothesis to be tested states that there is no relationship between tumour FMISO uptake and circulating OPN levels.

- Explore the possibility of using tumour biopsies to assess changes in hypoxia and perfusion from baseline to the end of the second week of CRT.

The ability of biopsies to identify rectal adenocarcinoma tissue, particularly during CRT, will be assessed. IHC of an endogenous marker of hypoxia (CAIX) and microvessel endothelial cells (CD31) will be used. The quality of ribonucleic acid (RNA) that can be extracted from

the rectal cancer biopsies will be investigated before moving forward with RNA sequencing to generate a rectal cancer-specific hypoxia metagene panel.

5.2 Methods

5.2.1 Trial design and patient selection

A clinical trial protocol was written, granted approval by a regional ethics committee (Essex Ethics Number 13/EE/0123) and is registered with clinicaltrials.gov (NCT02157246). The trial design of the RHYTHM (modulation of Radiotherapy according to Hypoxia: exploiting changes in the Tumour Microenvironment to improve outcome in rectal cancer) study can be seen in Figure 46. The study is divided into two cohorts, A and B, each with different aims. Cohort A explores the test-retest variability of FMISO-PET and pCT and validates FMISO-derived hypoxic volumes against whole-mount histology of pimonidazole staining. Cohort B is primarily concerned with characterising changes in hypoxia and perfusion from baseline to the end of the second week of CRT.

5.2.1.1 Cohort A

Patients with T2-3 N0-2 M0 histologically proven adenocarcinoma of the rectum with MRI confirmation that the circumferential resection margin is not involved or threatened and agreement from the local multidisciplinary team (MDT) that the tumour is operable and does not require preoperative CRT were eligible for study recruitment. Patients with resectable, histologically confirmed rectal adenocarcinomas that measured at least 2 cm x 2 cm were eligible to participate as long as there were no contraindications to the imaging studies or tumour biopsy.

5.2.1.2 Cohort B

Patients with locally advanced histologically confirmed invasive adenocarcinoma of the rectum that required neoadjuvant CRT were eligible for study entry. LARC is defined as cancer seen to be involving or threatening the resection margin on MRI. In short, patients with non-metastatic locally advanced rectal adenocarcinomas who were deemed to require downstaging preoperative CRT by the MDT were eligible for entry into Cohort B of the RHYTHM study.

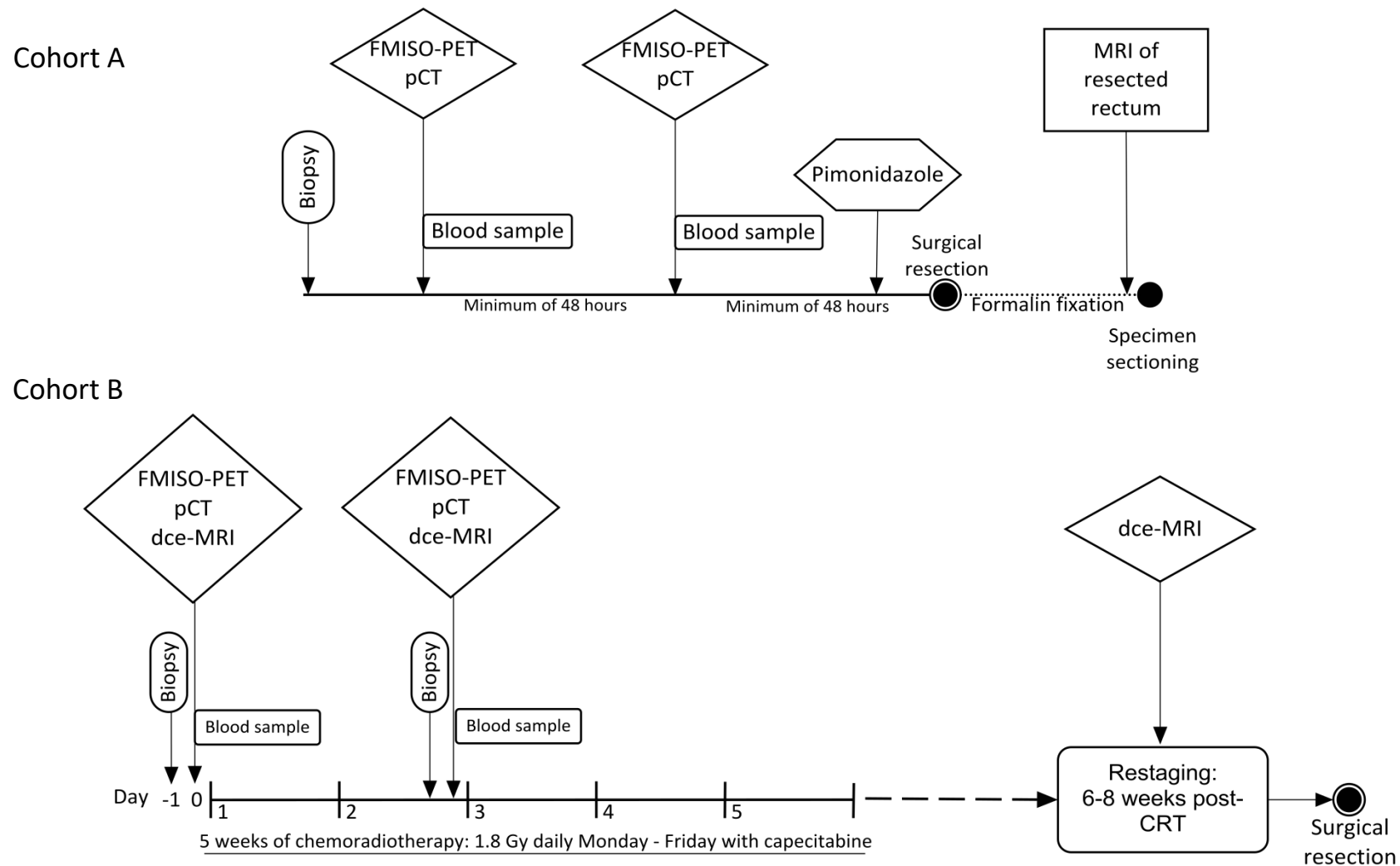


Figure 46: The RHYTHM study (Cohorts A and B) trial design.

The diagnostic, staging MRI scan that is standard of care for all patients is not included in this diagram, but was used for image registration purposes.

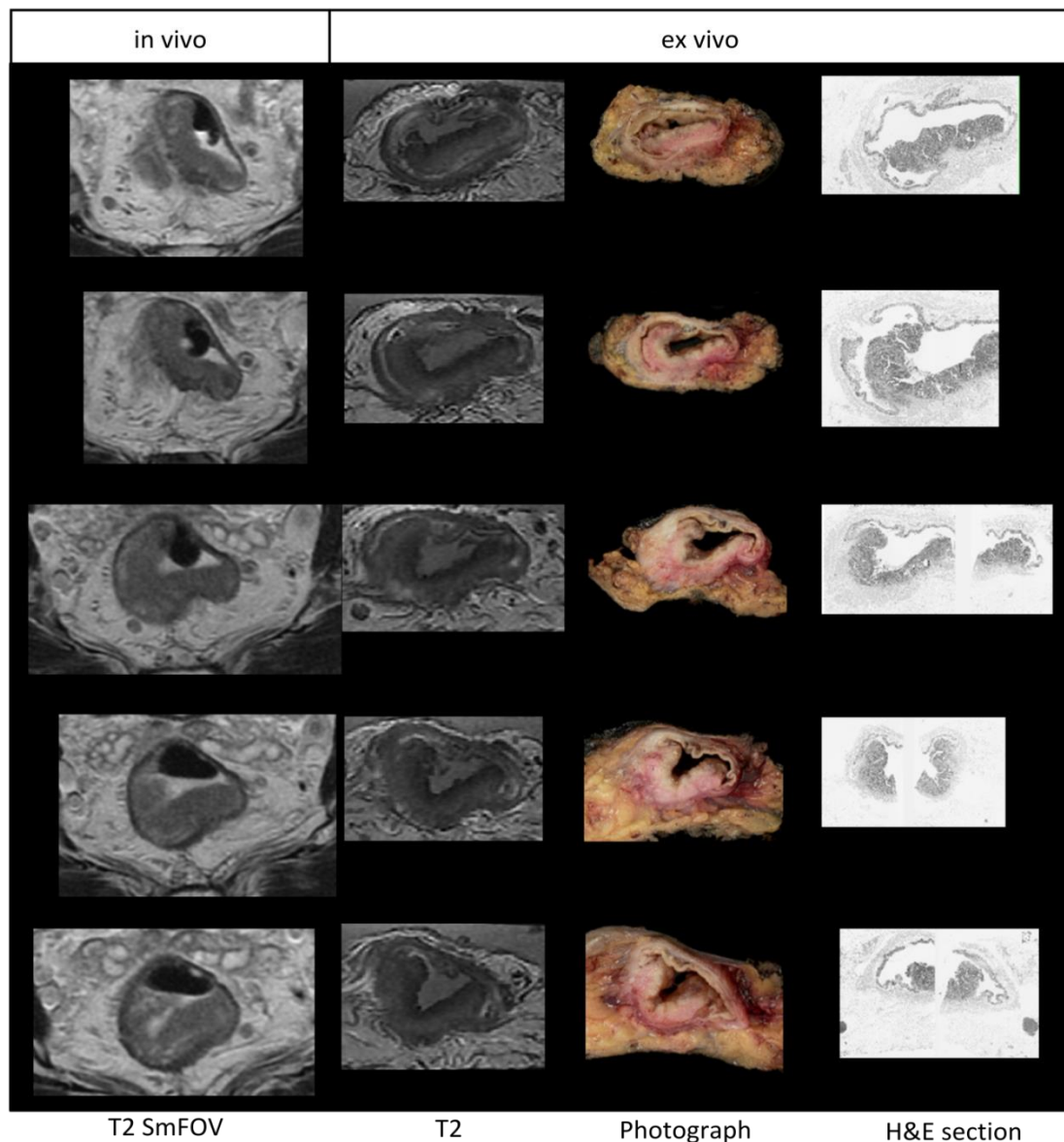
5.2.2 Trial interventions: Cohort A

All additional investigations were performed in the 7 days leading up to surgery. Once informed consent had been obtained, tumour biopsies were taken under direct visualisation at proctoscopy. No earlier than the next day, an FMISO-PET and pCT were performed. A minimum of 48 h later, the FMISO-PET and pCT were repeated. Blood was taken and stored at each imaging visit. Pimonidazole tablets (oral Hypoxyprobe™-1, Hypoxyprobe Inc., Burlington, MA, USA) at a dose of 500 mg/m² were taken between 16 and 24 h prior to surgical excision.

5.2.2.1 Sample handling of the resected rectum

Further details of the method that was developed to aid accurate image registration of whole-mount histology sections with *in vivo* imaging can be found in the RHYTHM trial sample handling manual. The rectal specimen was taken at the time of resection in the operating theatre, orientated in the anatomical position and pinned on corkboard before suspension in 10% formalin. The specimen was fixed in formalin overnight. The next day, the container containing the rectal resection specimen and formalin was imaged using MRI (GE 1.5 T Twinspeed HDx, Milwaukee, WI, USA, using an 8-channel GE body coil. Post localising and calibration scans, T2-weighted axial images were obtained at 3 mm intervals of the whole specimen). A pathologist then removed the specimen from the formalin and marked it for sectioning while referring to the MRI with an experienced radiologist. The section was then sectioned at 3 mm intervals in line with the MRI imaging and photographed before embedding in paraffin as per standard laboratory practice. A method of preparing the slides to allow for automated IHC staining using the Leica Bond-Max (Leica Biosystems, Milton Keynes, UK) can be seen in the sample handling manual.

Prior to study initiation, this specimen-handling pathway was tested in a fully consenting non-trial patient. The results of this test-run proving feasibility of the sample handling and imaging pathway as well as testing the ability to obtain accurate cross-modality images that can be matched across the whole TV can be seen in Figure 47.



Direction of image acquisition

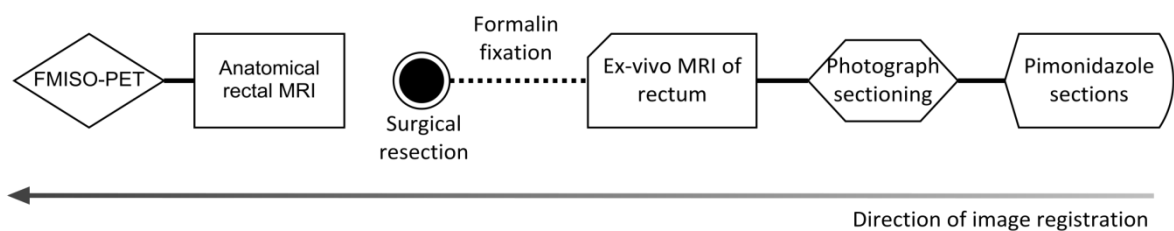


Figure 47: *In vivo* and *ex vivo* imaging of a rectal tumour acquired using the sample-handling procedure developed for RHYTHM A. The acquisition of images at each step of the sample-handling steps aims to provide as much imaging data as possible to aid accurate image registration of pathology slides with *in vivo* hypoxia and perfusion imaging. All images are separated by 3 mm sections. It is anticipated that this will require multiple registration steps. The direction of image acquisition and how sequential image registrations may be used to accurately compare pimonidazole staining against FMISO-PET-derived hypoxic volumes is also shown. (T2SmFOV = T2-weighted MRI, *in vivo* image. T2 = T2-weighted MRI, *ex vivo* image)

5.2.3 Trial interventions: Cohort B

Once patient consent had been obtained, tumour biopsies were taken. The next day, dce-MRI, FMISO-PET and pCT were performed. Every effort was made to ensure the biopsies and imaging were taken as close to the start of CRT as possible. Tumour biopsies were repeated once the patient had received between 8 and 10 fractions of radiotherapy. The dce-MRI, FMISO-PET and pCT were repeated the day following the second biopsy. Blood samples were taken on the same day as the imaging studies. At the end of the CRT delivery, the total dose of capecitabine and radiotherapy was documented. At the same time as routine clinical restaging investigations, a repeat dce-MRI was performed. The patient went on to have a surgical resection as per routine clinical care. Results of restaging investigations and histopathological response to CRT were recorded.

5.2.3.1 Chemoradiotherapy schedule for patients in Cohort B

Patients received capecitabine 900 mg/m² twice daily concurrently with pelvic radiotherapy consisting of 45 Gy in daily 1.8 Gy fractions over 5 weeks on Monday to Friday. This regimen was selected as it is the standard treatment arm in a currently recruiting national Phase III trial (ARISTOTLE – EudraCT number 2008-005782-59). Radiotherapy was delivered according to a standard departmental protocol using a 5- to 7-field intensity-modulated radiotherapy technique. The rectal tumour, mesorectum and pelvic lymph nodes were included in the radiotherapy planning target volume.

5.2.4 Imaging acquisition protocols

All images were acquired with the patient positioned on a flat couch with knee rests and ankle stocks, with alignment of skin tattoo markers with an indexed, wall-mounted laser system to replicate the radiotherapy treatment position to aid accurate image registration.

5.2.4.1 *¹⁸F-fluoromisonidazole-PET/CT*

The imaging protocol as described in Chapter 3 was used. In short, the PET/CT was acquired on a GE Discovery 690 scanner. The patient was aligned using permanent skin-marking tattoos and a room-mounted laser system. CT was performed to localise the rectal tumour (120 kV automA (max 250 mA), noise index 25.0 0.5 s/rotation, pitch 0.984:1, 3.75 mm slice width). FMISO 370 MBq was injected as a bolus 30 s after starting the PET acquisition. The dynamic PET sequence was acquired in variable time frames; 12 x 5 s followed by 6 x 10 s, 5 x 30 s, 10 x 60 s and 6 x 5 min frames (a total acquisition time of 45 min). A CT scan followed by a 10 min static acquisition PET image and was then acquired 2 and 4 h post FMISO injection with the patient in the same radiotherapy treatment position. Arteriovenous blood was taken 4 min, 60 min, 2 h and 4 h post FMISO injection (using a method developed within the department that uses hand warming to increase venous dilatation on the hand to allow 'arteriovenous' blood sampling) to be used later to correct an image-derived AIF.

5.2.4.2 *Perfusion CT*

Perfusion CT was performed immediately following the 4 h acquisition of the FMISO-PET with the patient in the same position on the GE Discovery 690 scanner. A pre-contrast CT was taken (100 kV, 130 mA, noise index 32.0 0.5 s/rotation, pitch 0.984:1, 6.25 mm slice width) to localise the tumour. The rectal tumour was localised and a 40 mm scanning area for the perfusion sequence was identified. When the length of the tumour exceeded 40 mm, the central point of the tumour was set to the mid-point of the field of view. An example of the resultant parameter map over the 40 mm field of view (FOV) and how that relates to the TV can be seen in Figure 48. Contrast (100 mL, Omnipaque 300, GE Healthcare) was injected over 45 s followed by a 50 mL saline flush, which was injected at the same rate. A continuous

acquisition with a 1.0 s rotation time and 8 images/rotation was obtained for a 45 s exposure (100 kV, 130 mA, 5.0 mm slice thickness).

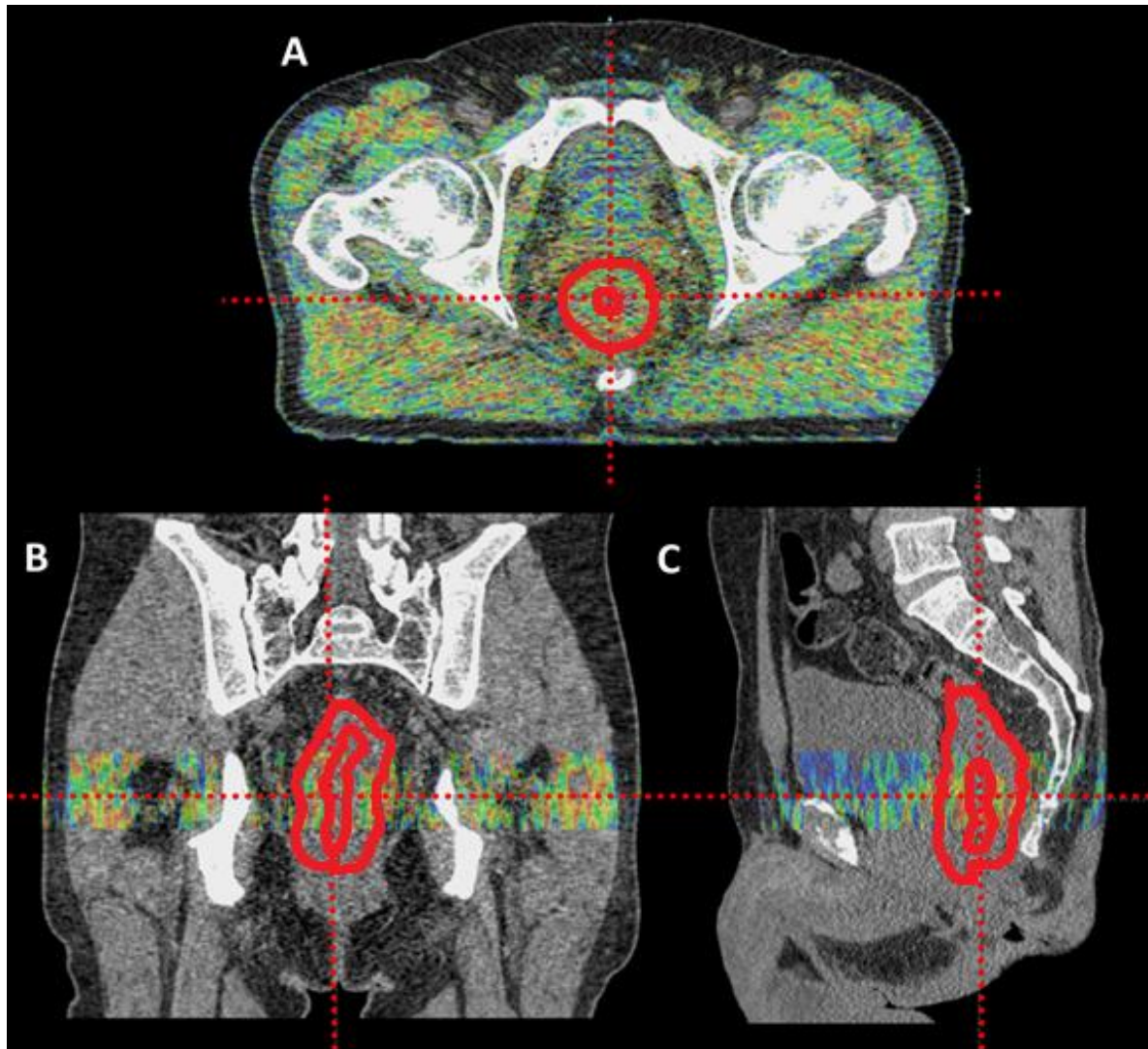


Figure 48: Axial (A), coronal (B) and sagittal (C) sections through a coregistered image of a pre-contrast CT and BF parameter map of a patient with LARC. The 4 cm field of view was placed in the centre of the tumour when the whole tumour volume (solid red line) could not be imaged.

5.2.4.3 *Dynamic contrast-enhanced MRI*

All patients were given hyoscine butylbromide 20 mg by intravenous injection prior to scanning. Scans were performed on a whole-body clinical MR system, a 1.5 T Twinspeed HDx (GE, Milwaukee, WI, USA) using an 8-channel GE body coil. Post localising and calibration scans, T2-weighted coronal and axial images were obtained on the region of interest. To

account for T1 inhomogeneity, three flip angles (3°, 9°, 15°) were acquired for post-processing T1 correction. A 3D LAVA sequence is used pre and post contrast of gadoteridol 20 mg/mL (Prohance®, Bracco UK Ltd, High Wycombe) at 3 mL/s, 0.2 mL/kg body weight with a FOV of 40 cm. A lower resolution, time-resolved T2*-weighted imaging sequence was used pre- and post a second contrast injection to observe contrast uptake and generate an AIF.

5.2.5 Tumour biopsies

Biopsies were taken via flexible sigmoidoscopy at the time-points indicated in Figure 47. Tumour material was obtained using Captura® biopsy forceps (Cook Medical Europe Ltd, Limerick, Ireland). Three biopsies were taken at each time-point. Each biopsy is large enough to cut in half. Half of the material from each biopsy was placed in formalin for sectioning and staining with haematoxylin and eosin (H&E) to confirm that invasive adenocarcinoma had been biopsied and to provide sections for IHC staining. The other half of each biopsy was placed in RNAlater® (Life Technologies Ltd, Paisley, UK) for RNA sequencing. H&E-stained sections of the biopsy were reviewed by an experienced pathologist. IHC for CAIX and CD31 and RNA extraction was performed only on samples that were confirmed to contain invasive tumour.

5.2.6 Immunohistochemistry

All staining protocols were optimised in the Oxford Cancer Centre Good Clinical Practice laboratory by Dr Leticia Campo. Once optimised, all IHC protocols were automated on the Leica Bond-Max (Leica Biosystems, Milton Keynes, UK). All protocols added the primary antibody for 30 min, post-primary antibody (Novocastra Bond Polymer Refine Detection Kit, Leica Biosystems) for 8 min, diaminobenzidine (DAB) for 10 min and haematoxylin for 5 min.

The antibodies used were:

- **Pimonidazole:** Hypoxyprobe-1 MAb1 anti-pimonidazole mouse IgG1 monoclonal antibody (NPI, Burlington, MA, USA) diluted to 1:1000 in dilution buffer
- **CAIX:** Rabbit polyclonal anti-CAIX (Abcam, Cambridge, UK) 1: 2000
- **CD31:** monoclonal mouse anti-CD31 JC70A (DAKO, Ely, UK) 1:800

Semi-quantitative assessment of staining was performed on a Leica DM550 B microscope (Leica Microsystems, Milton Keynes, UK). Slides were also imaged using an Aperio Scanscope slide scanner at 20x magnification. Tiled images were then reconstructed and exported for automated quantification.

5.2.7 RNA extraction for sequencing

RNA was extracted from the tumour biopsies that had been stored in RNAlater® by Dr Su Lim (Professor Muschel's laboratory) using a protocol that can be found in Appendix C. The quality of the resected RNA was checked before sending the extracted RNA for sequencing.

5.2.8 Image analysis

5.2.8.1 ¹⁸F-fluoromisonidazole-PET/CT

Maximum and peak (mean activity within the 1 cm³ sphere with the highest activity) FMISO uptake values were obtained from the attenuation-corrected, static acquisition PET/CT acquired 4 h post tracer injection (4 h FMISO-PET). The CT component of the PET was rigidly registered to the contrast-enhanced CT used for radiotherapy planning in Eclipse (version 13, Varian Medical Systems, Palo Alto, CA, USA).¹⁹⁷ The GTV had already been delineated on the contrast-enhanced planning CT after image registration of the planning CT with the T2 MRI. The GTV was then propagated to the CT component of the PET to generate a ROI labelled as

‘tumour’. The gluteal muscles and lumen of the external iliac arteries were outlined on all tumour-containing slices of the CT and were labelled ‘muscle’ and ‘blood’ respectively.

The 4 h PET and the ROIs were imported to PMOD software (PMOD Technologies, Zurich, Switzerland).¹⁹⁸ The SUV_{max} , SUV_{peak} , $T:M_{max}$ and $T:B_{max}$ were calculated at each time-point.

To define a subvolume of the tumour that could be described as ‘hypoxic’, the volume of tumour with an uptake value greater than reference tissue was used. Two previously recognised thresholds of FMISO uptake, the same as were used in pancreatic cancer in Chapter 3, that are thought to represent hypoxia were used: a $T:M > 1.3$ ^{95,170-172} and a $T:B > 1.4$.^{153,177,178,183} The volume of tumour with FMISO uptake greater than these thresholds was calculated on all FMISO-PET images.

5.2.8.2 Perfusion CT

Perfusion parameter maps were generated using the GE Advanced Workstation 4.0 and the ‘body’ perfusion protocol, using the same method as described in Chapter 3 with the exception that the external iliac arteries were used to generate an AIF rather than the aorta. The same ‘tumour’ ROI used for FMISO uptake quantification was used to analyse the pCT parameter maps. The pre-contrast component of the pCT was rigidly registered to the contrast-enhanced planning CT using Mirada (Mirada Medical, Build 1.0.1.4, Oxford, UK). The tumour ROI was then propagated on to the pre-contrast CT. The pre-contrast CT shares the same frame of reference as the pCT parameter maps. The tumour ROI volume could then be transferred to the parameter maps to allow for mean BF and BV to be calculated in Mirada.

5.2.8.3 *Dynamic contrast-enhanced MRI*

Tumour ROIs were defined on the T2-weighted small FOV MRI sequence of the dce-MRI using ITK-SNAP (www.itksnap.org). Pharmacokinetic parameters from the perfusion MRI sequence were obtained using in-house software developed by Monica Enescu (Professor Julia Schnabel's group). This method is described in detail elsewhere.²⁶⁵ In short, the software first registers the tumour ROI delineations to the first image (pre-contrast) in the perfusion sequence. The perfusion sequence is then corrected for motion using intrasequence registrations based on a Markov random field (MRF). The MRF is a graphical representation of a joint probability function. It assumes that all 'nodes' within the graphical distribution function are random variables. This approach defines voxels, or groups of voxels, within the images as 'nodes'. For each node, a probability function for the potential random displacement of the node is generated. A deformable image registration between images in the contrast-enhanced series can then be performed by optimising the displacement of each node in pair-wise fashion. The full 4D information within the image based on contrast enhancement and washout is used in the pairing of nodes to aid accurate image registration and therefore motion correction. For more details of this method, see Enescu *et al.*²⁶⁵ Pharmacokinetic parameter estimation is performed after motion correction using the standard Tofts model.²⁶⁶ K_{trans} , a parameter that quantifies the movement of tracer from the circulation to the extracellular space, was used as a marker of perfusion.

5.2.8.4 *Immunohistochemistry of pimonidazole, CAIX and CD31*

Firstly, a validated semi-quantitative scoring was performed. Slides were viewed with an experienced histopathologist (Dr Lai Mun Wang) and scored independently. When scores differed, slides were viewed together and a consensus was reached.

Pimonidazole staining from whole-mount histology sections of the resected rectum from patients in cohort A was quantified using a recognised visual scoring system that is known to have excellent interobserver reproducibility.^{126,267} Pimonidazole staining was scored based on the volume of tissue with positive staining as: 0, no staining; 1, >0–5%; 2, >5–15%; 3, >15–30%; 4, >30%. A visual scoring system of CAIX staining that has been validated and proven to be prognostic was used.^{129,268} CAIX staining was scored as follows: 0, negative staining in all cells; 1+, weakly positive or focal staining in <10% of cells; 2+, moderately positive staining in >10% of cells; 3+ strongly positive in >10% of cells.¹²⁹ To quantify microvessel density, the Chalkley counting method was applied.²⁶⁹ The Chalkley counting method was used to quantify CD31 staining as it is reproducible, is comparable to other microvessel counting methods and has been shown to be prognostic.²⁷⁰ The number of Chalkley graticule contact points with CD31-positive areas was counted three times in different parts of each section and a mean of the counts was taken.

An additional method was developed to automate the quantification of each type of DAB staining of the slides of the biopsy material from cohort B. ImageJ (NIH, Bethesda, MD, USA) was used to first spectrally unmix the image by converting the blue spectrum pixels (haematoxylin) to a green image and the DAB (brown) pixels to red. The green and red channels could then be split. The green image was segmented using the auto-threshold 'Huang' to generate a whole-tissue area mask that could be measured. The red (DAB) image was then overlaid on the tissue mask and the area of red tissue within the mask was calculated. The area of red pixels (DAB) was then expressed as a percentage of the total tissue area. An example of the original and spectrally unmixed images can be seen in Figure 49. Any

change of <10% in the area of the biopsy that was staining positive for either CD31 or CAIX was labelled as 'unchanged'.

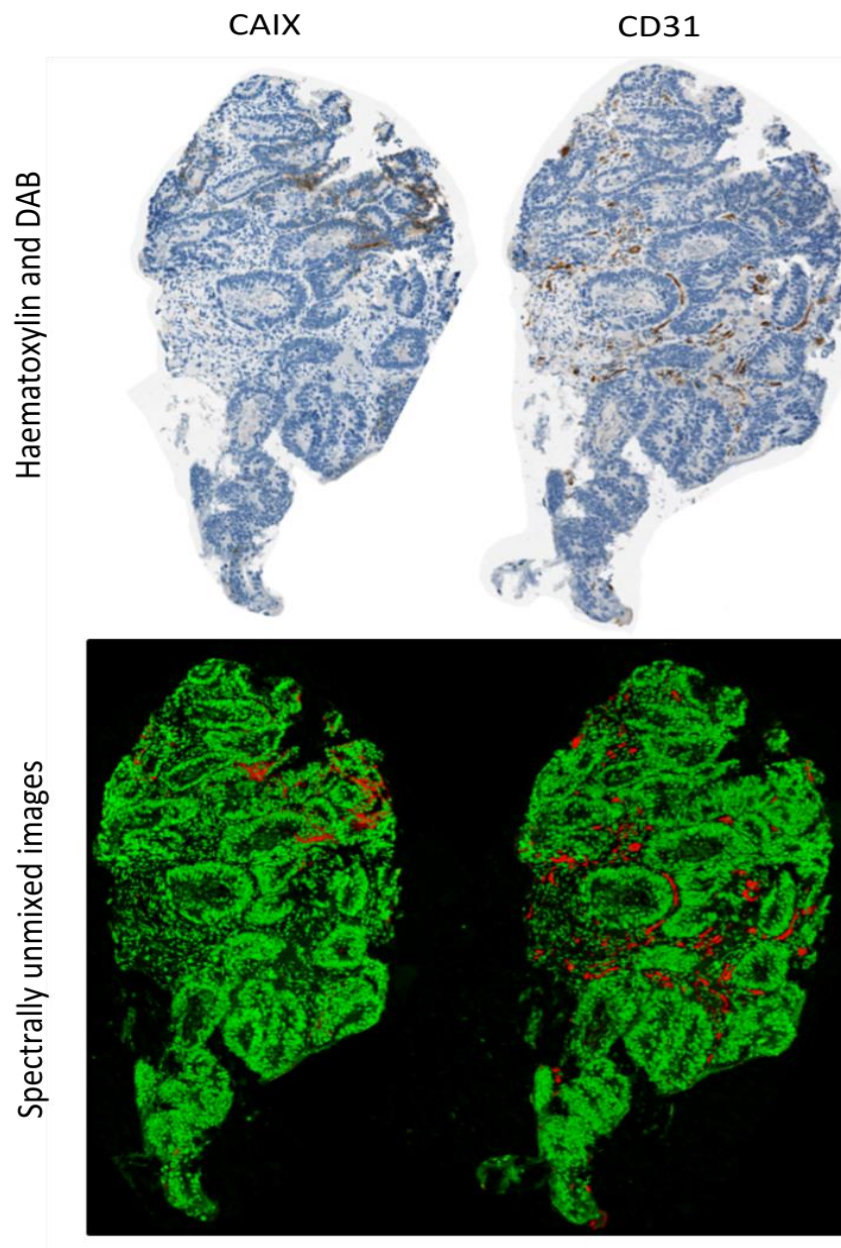


Figure 49: Haematoxylin and DAB staining and post-processing images of CAIX- and CD31-stained sections of biopsy material from a patient in Cohort B.

5.2.9 Histological tumour regression grade

H&E-stained sections from the resected rectum were examined by an experienced pathologist (Dr Lai Mun Wang) who was blinded to all imaging data. Response was evaluated

using the Mandard TRG.⁵² The Mandard TRG, as discussed in Chapter 1, is scored as follows.

1: pCR; 2: residual cancer cells scattered through fibrosis; 3: an increased number of residual cancer cells with predominant fibrosis; 4: residual cancer outgrowing fibrosis; 5: no regressive changes within the tumour.

5.2.10 Defining radiological response

MRI tumour regression grade (mrTRG) was assessed by an experienced radiologist based on the degree of post-CRT fibrosis as previously described.²⁷¹ (In short, mrTRG-1: absence of tumour signal; mrTRG-2: small amounts of residual tumour signal with predominant fibrotic low signal intensity; mrTRG-3: mixed fibrosis and tumour signal; mrTRG-4: predominantly tumour signal with minimal fibrotic low intensity signal; mrTRG-5: no evidence of fibrosis, tumour signal only.)

5.2.11 Osteopontin quantification

Blood was taken on the same day as FMISO-PET imaging as outlined in Figure 46. The blood sample was processed in the Oxford Cancer Centre Good Clinical Practice laboratory as outlined in Appendix C. Serum OPN concentration was then ascertained using the Human Osteopontin Magnetic Luminex Performance Assay (R&D Systems, Abingdon, UK).

5.3 Results

5.3.1 Patient demographics

To date, one patient has been recruited into Cohort A of the RHYTHM study and 5 patients have been recruited and completed the imaging and required follow-up in Cohort B. A 49-year-old male patient with T3 N0 M0 rectosigmoid adenocarcinoma was recruited to Cohort A. Patient characteristics of the RHYTHM B participants are outlined in Table 20. One of the patients did not go on to have surgical resection post CRT. This was in part due to patient

preference and also because of a good radiological response on post-CRT restaging investigations.

Table 20: Demographics of patients recruited to RHYTHM Cohort B

Patient	Age	Gender	TNM stage	Tumour volume (cm ³)	TME post CRT
A	61	M	T3 N1 M0	80.4	Yes
B	61	M	T3 N1 M0	22.7	Yes
C	62	M	T3 N0 M0	178.6	Yes
D	74	M	T3 N0 M0	29.8	Yes
E	85	M	T3 N1 M0	62.1	No

5.3.2 RHYTHM Cohort A

5.3.2.1 Registration of tumour histology sections with *in vivo* imaging

The imaging schedule of the resected tumour allowed the successful identification and linkage of the pimonidazole-stained section with the *in vivo* and *ex vivo* MRI images and the cut section photograph. Matching of images from one axial section of the tumour can be seen in Figure 50.

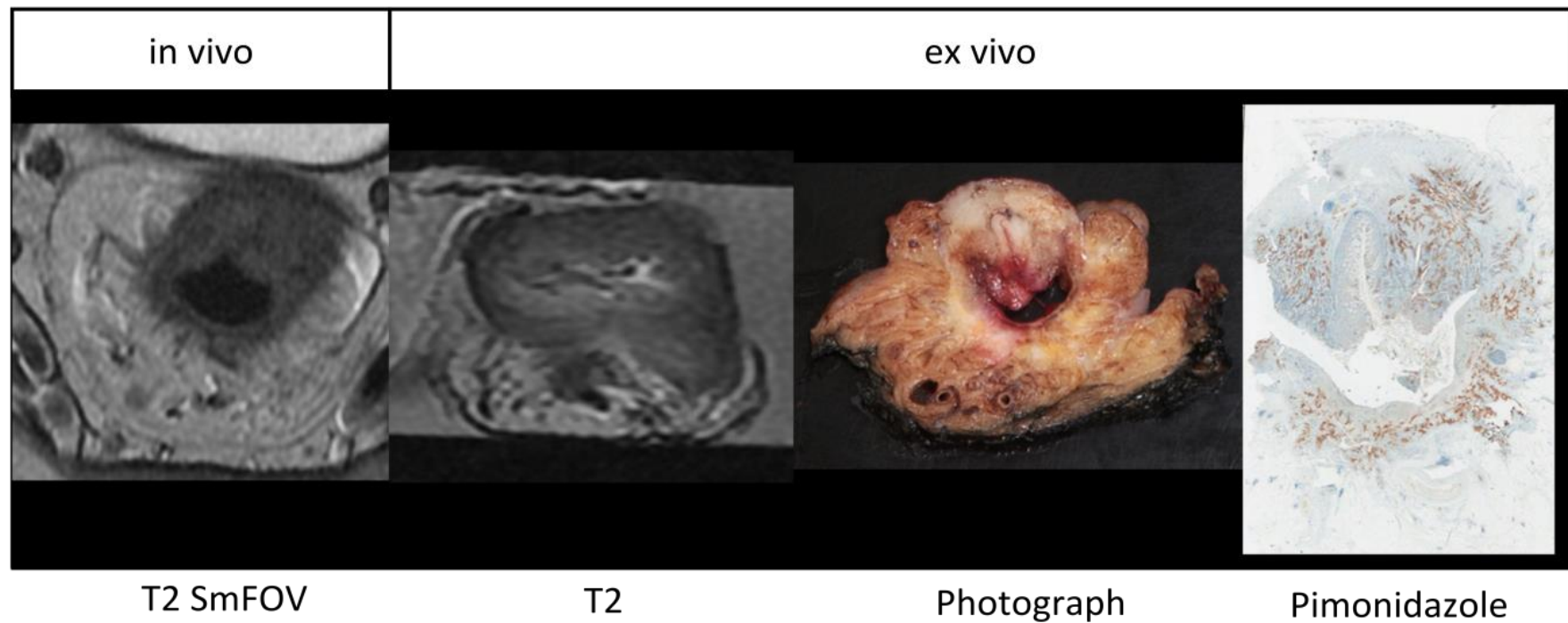


Figure 50: In vivo and ex vivo imaging, including a pimonidazole-stained section from the first patient recruited to Cohort A of the RHYTHM study (T2 SmFOV = T2-weighted small field of view image from the diagnostic MRI scan, T2 = T2-weighted MRI of the resected rectal tumour).

In collaboration with Andre Hallack Miranda Puereza (Professor Julia Schabel's group, Biomedical Engineering), some progress has been made in developing computer software that will ultimately allow the accurate registration of IHC-stained tumour sections with *in vivo*, pre-resection imaging. A process that allows the registration of tumour sections with the *ex vivo* MRI is almost completed. An example of the image registration that is currently possible can be seen in Figure 51.

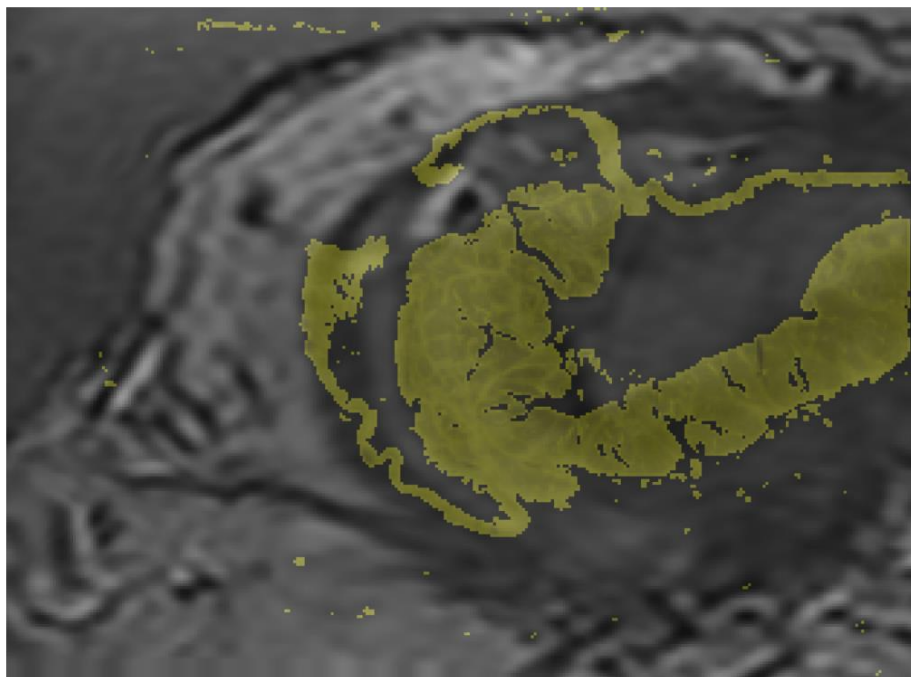


Figure 51: Registration of a 'mask' of the tumour tissue outline of a histology tumour section (yellow) with the T2-weighted MRI of the resected rectum. Some alignment of the mucosa can be seen, but the algorithm needs to be refined to improve accurate registration of the tumour. (Image provided by Andre Hallack Miranda Puereza)

5.3.2.2 ^{18}F -fluoromisonidazole-PET/CT

The FMISO-PET images acquired 4 h post tracer injection on both Day 0 and the repeat scan on Day 3 of study participation can be seen in Figure 52. It was not possible to quantify tumour FMISO uptake in either of the scans for this patient because of spill-over from activity from FMISO within the rectal lumen. It can also be seen that high activity within the bladder also

spills over into the tumour ROI further complicating accurate quantification of tumour FMISO retention on either image.

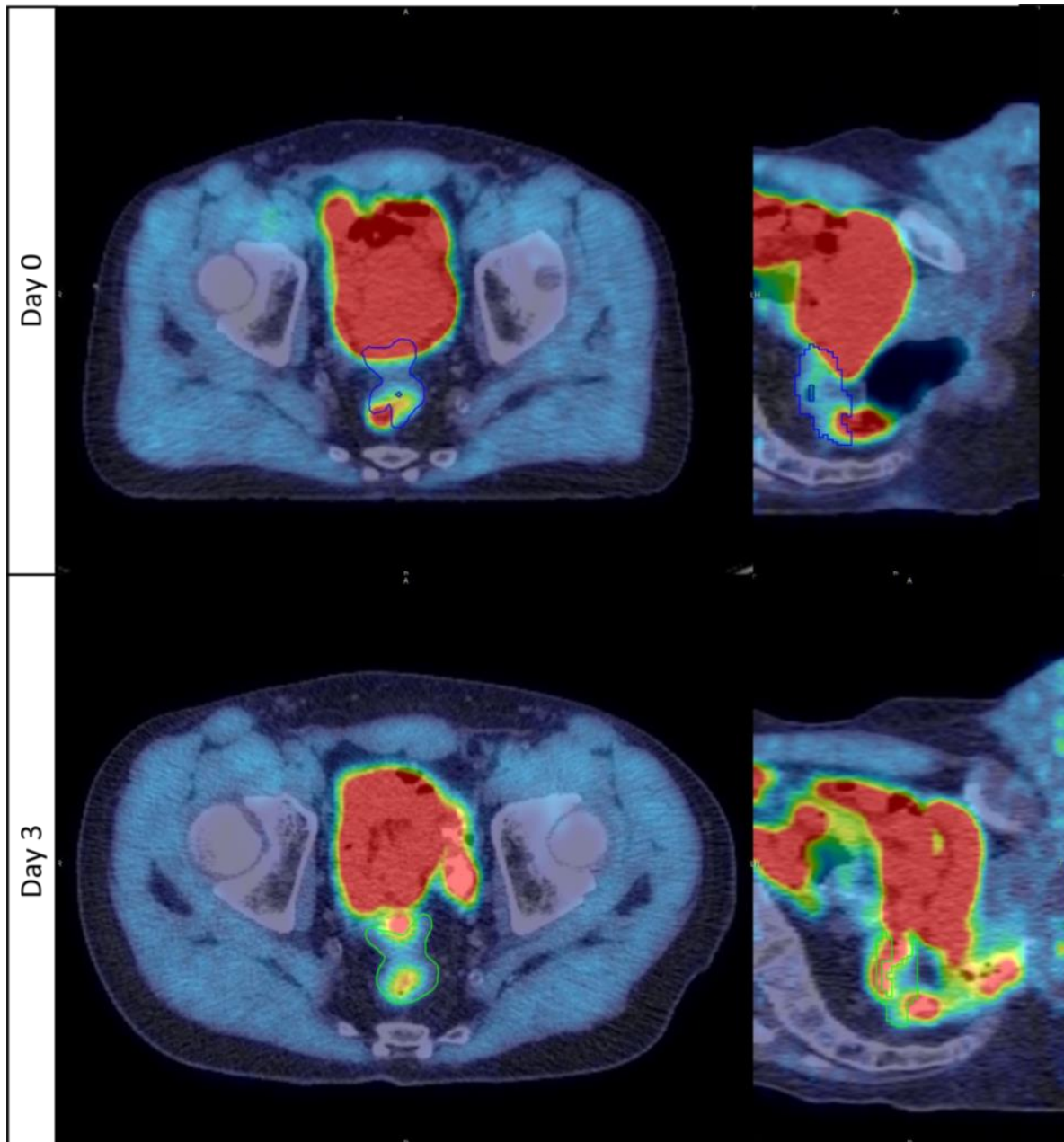


Figure 52: Axial and sagittal sections from an FMISO-PET/CT acquired 4 h post tracer injection in a patient from Cohort A of the RHYTHM study. The tumour has been outlined on both images (Day 0, blue; Day 3, green). Tracer signal can be seen in the lumen of the small and large bowel extending to the rectum. Spill-over from luminal contents and bladder made it impossible to assess tumour uptake at both time-points.

5.3.2.3 Perfusion CT

Mean tumour BF was 63.9 and 54.2 mL/100 g/min on scan 1 and scan 2 respectively, giving a variation of 16.4%. Mean tumour BV was 6.8 and 8.6 mL/100 g on the two separate scans, showing a variation of 23.4%.

5.3.2.4 Pimonidazole staining of tumour sections

All eight of the tumour bearing sections of the resected rectum had significant pimonidazole staining. Every tumour section was scored 4 (>30% staining positive for pimonidazole). An example of a DAB-stained section for pimonidazole can be seen in Figure 50.

5.3.3 RHYTHM Cohort B

Pre-CRT images were acquired the day before starting CRT in all 5 of the patients. The repeat imaging was taken after the patient had received 10 fractions of radiotherapy in all of the patients. Tumour biopsies were taken the day before the imaging in each of the patients (Day -2 and after 9 fractions of radiotherapy). Results concerning the characterisation of tumour hypoxia will be presented followed by quantification of tumour perfusion. All biopsies were reviewed with a histopathologist. Only tumour-bearing biopsy material was used for subsequent analyses. All patients had at least one biopsy that contained invasive adenocarcinoma at baseline and the end of 2 weeks of CRT.

5.3.3.1 ¹⁸F-fluoromisonidazole-PET/CT

Further problems were encountered with FMISO being present within the rectal lumen making tumour-uptake quantification impossible in three of the 10 FMISO-PET images. An example of an FMISO PET image from a patient recruited to Cohort B with evidence of the colonic excretion of FMISO reaching the rectum by 4 h post injection can be seen in Figure 53.

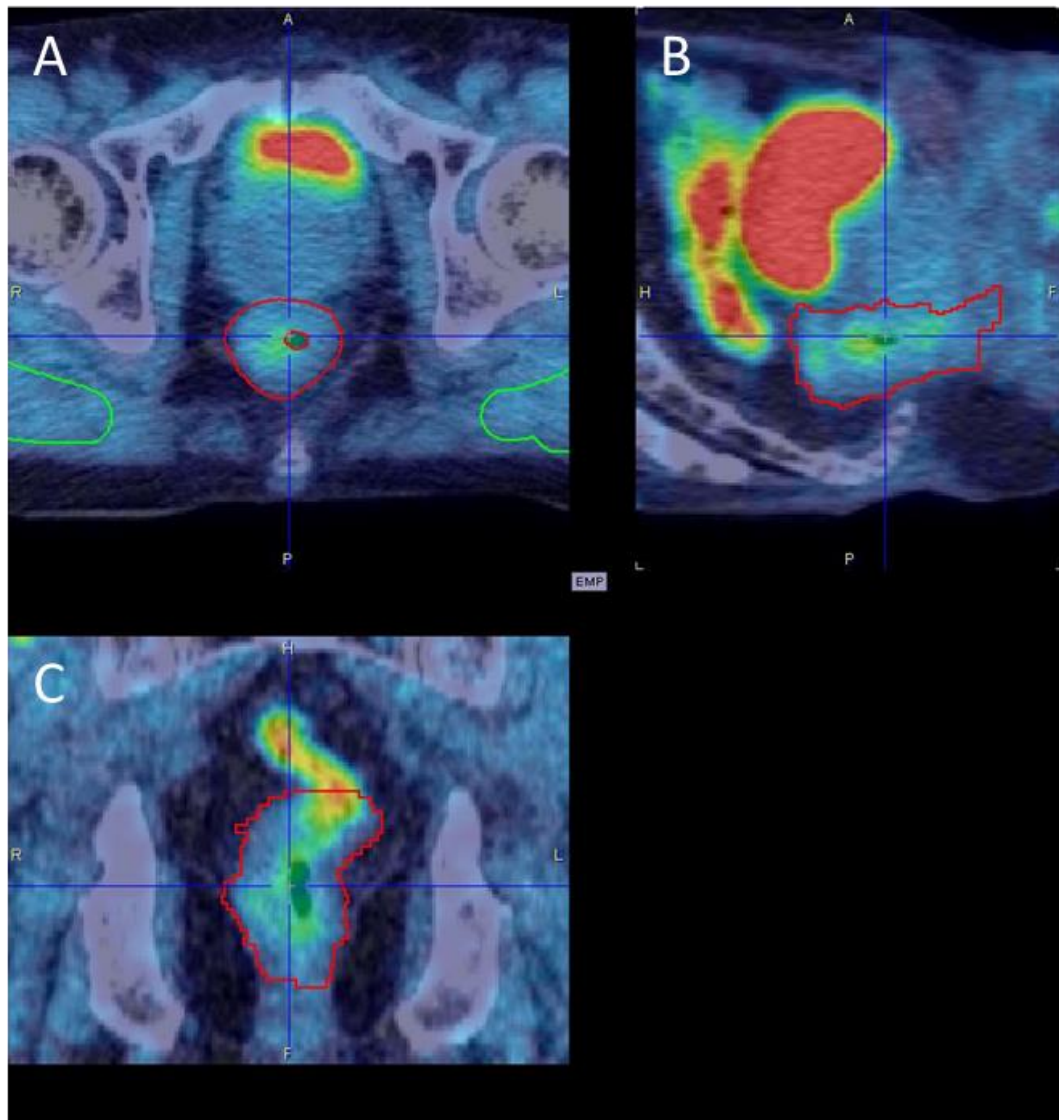


Figure 53: Axial (A), sagittal (B) and coronal (C) sections of an FMISO-PET/CT acquired 4 h post tracer injection. Entry of FMISO into the rectum from the colon is most clearly demonstrated in the coronal section (C). The tumour region of interest is outlined in red.

It was possible to quantify the FMISO uptake in the rectal tumour in the remaining seven FMISO-PET images. Results of the SUV_{max} , $T:M_{max}$ and $T:B_{max}$ and the FMISO uptake volume as defined by a $T:M > 1.3$ or $T:B > 1.4$ can be seen in Table 21. In the 3 patients with assessable paired imaging, the maximum uptake values and hypoxic volume was seen to decrease in 2 patients and increase in one patient. The FMISO-defined 'hypoxic volumes' ranged from 1.0 to 45.0% of the GTV at baseline and 1.1 to 26.8% of the GTV at the end of the second week of CRT when a $T:M > 1.3$ was used to define hypoxia. When, a $T:B > 1.4$ was used, these ranges

were 3.2–59.1% of the GTV and 2.2–14.1% of the GTV pre-CRT and at the end of the second week of CRT respectively. In the one patient in whom the hypoxic volume increased from baseline to the end of the second week of CRT, the FMISO uptake volume increased by 20% when T:M was used to define hypoxia or 93.6% when T:B was used. The absolute uptake volume in this patient was small ($\leq 5.1 \text{ cm}^3$ or $\leq 6.3\%$ of the GTV). The 2 patients who demonstrated a reduction in FMISO-defined hypoxia had a >76% reduction in the hypoxic volumes defined using the different tumour-to-background thresholds.

An example FMISO-PET/CT image from a patient who had a reduction in the FMISO uptake volume and one patient who had an increase in FMISO retention can be seen in Figure 54. In all three of the patients with evaluable paired imaging, the region of FMISO retention was spatially consistent between the two time-points on visual assessment of the imaging. It can also be seen that there has not been a reduction in GTV from baseline to the end of the second week of CRT.

Table 21: Quantification of FMISO uptake pre-CRT and at the end of the second of week of CRT (2wk) in the first 5 patients recruited to RHYTHM Cohort B. The uptake volume using a T:M and T:B can be seen.

Patient	Tumour volume (cm ³)	SUV _{max}		T:M _{max}		T:B _{max}		T:M > 1.3 volume (cm ³)		T:B > 1.4 volume (cm ³)		% change in FMISO uptake volume	
		Baseline	2wk	Baseline	2wk	Baseline	2wk	Baseline	2wk	Baseline	2wk	T:M > 1.3	T:B > 1.4
A	80.4	2.0	2.2	1.4	2.2	1.8	2.6	0.8	0.9	2.6	5.1	20.0	93.6
B	22.7	*	*	*	*	*	*	*	*	*	*		
C	178.6	3.0	2.1	2.4	1.6	2.8	2.0	48.0	0.5	63.2	4.0	-99.0	-93.7
D	29.8	3.5	2.5	2.8	2.1	3.3	2.4	13.4	2.4	17.6	4.2	-82.0	-76.5
E	62.1	*	1.8	*	1.4	*	1.7	*	0.6	*	1.5		

* = Significant rectal luminal FMISO making quantification of tumour FMISO uptake impossible.

Table 22: A: Quantification of CAIX staining of biopsy material taken pre-CRT and at the end of the second week of CRT. Two methods of quantifying the CAIX staining have been used. B: Changes in serum OPN levels.

A		Semi-quantitative ¹²⁹			Quantitative (% of tissue +ve)				B	Osteopontin (ng/mL)			
Patient		Baseline	2 wk	Δ	Baseline	2 wk	%Δ	Δ		Baseline	2 wk	%Δ	Δ
A		0	0	↔	3.8	0.7	-81.6	↓		56.3	65.6	16.5	↑
B		2+	1+	↓	4.8	1.1	-77.1	↓		85.7	73.1	-14.7	↓
C		0	0	↔	0.7	0.7	0.0	↔		65.7	56.6	-13.9	↓
D		1+	0	↓	0.9	0.6	-33.3	↓		85.2	76.3	-10.4	↓
E		1+	1+	↓	0.6	2.9	383.3	↑		41.1	56.2	36.7	↑

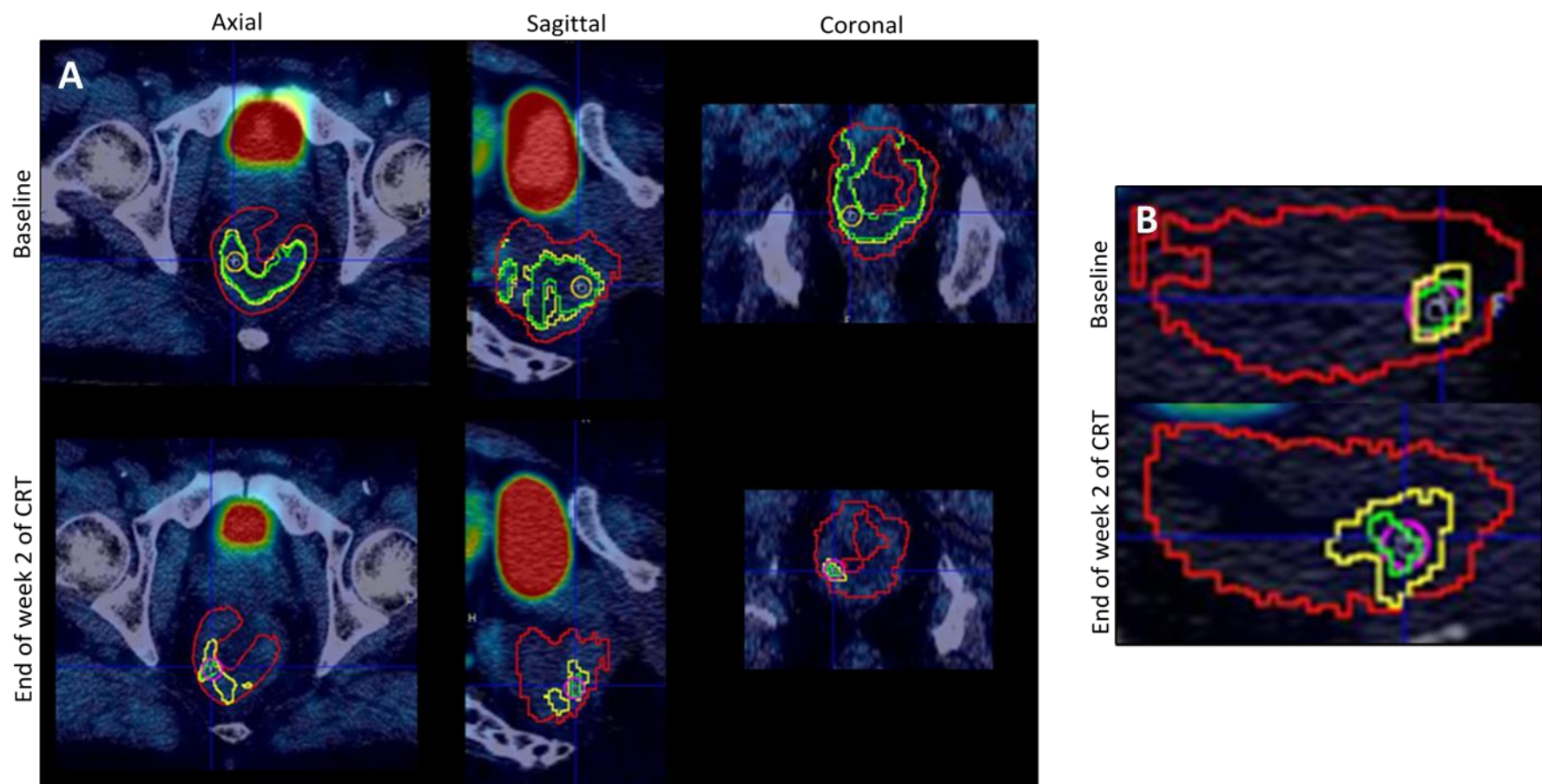


Figure 54: Example FMISO-PET/CT images from Cohort B of the RHYTHM study. Panel A shows axial, sagittal and coronal images from FMISO-PET/CTs taken at baseline and at the end of the second week of CRT. The FMISO uptake volume has decreased. Panel B shows sagittal sections from an FMISO-PET/CT in which the FMISO uptake volume is seen to increase from baseline to the repeat at the end of the second week of CRT. (Yellow = T:M > 1.3, Green = T:B > 1.4 isocontours. The SUV_{peak} sphere can also be seen on each image, pink). The MRI-defined GTV is shown in red.

5.3.3.2 CAIX staining of the rectal cancer biopsy material

The quantification of CAIX on the rectal cancer biopsy material taken pre-CRT and at the end of the second week of CRT is summarised in Table 22. Using the validated, semi-quantitative method, CAIX staining was shown to decrease in 3 out of 5 patients. It increased in one patient and did not change in one patient. Examples of sections of biopsy that scored 0, +1 and +2 for CAIX using this method can be seen in Figure 55. The automated method that calculated the percentage of the tissue area that stained positively for CAIX also demonstrated a decrease in CAIX staining in 3 of the 5 patients, with CAIX staining increasing in one patient and unchanged in another. The direction of change in the CAIX quantification using the different methods agreed in only 2/5 patients.

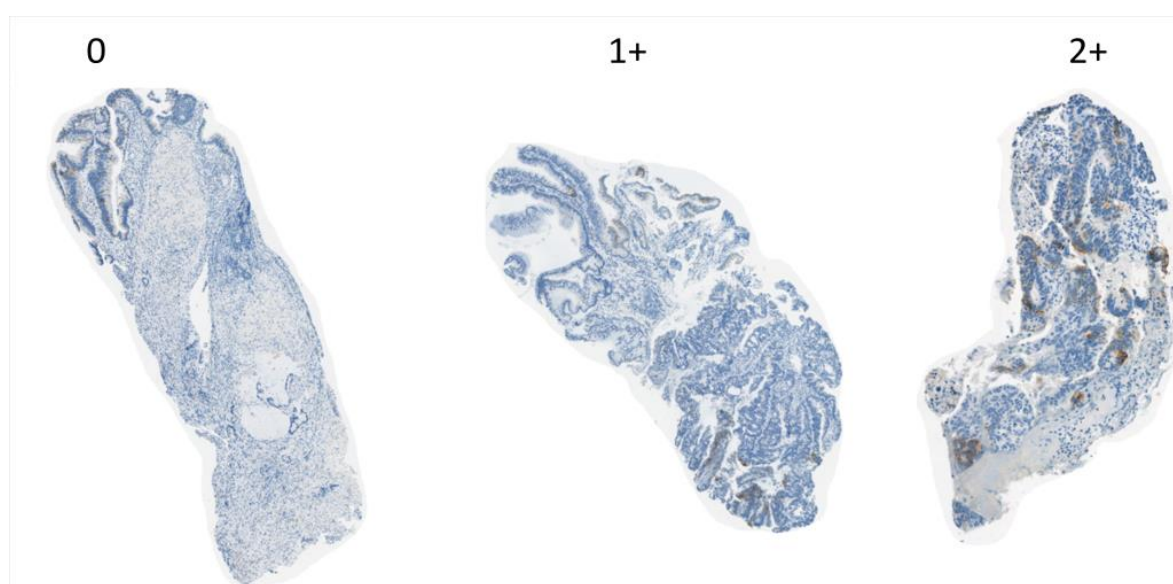


Figure 55: DAB staining of rectal cancer biopsies for CAIX. Examples given represent different CAIX scores using the described semi-quantitative method.

5.3.3.3 Osteopontin

Serum OPN levels decreased in 3 of the 5 patients and increased in the remaining 2 patients. The magnitude of change ranged from 10.4 to 36.7% of the baseline serum concentration

value (see Table 22(B)). The direction of change in OPN agreed with the direction of change in FMISO uptake volumes in each of the patients with interpretable paired FMISO imaging.

5.3.3.4 Perfusion CT

Results of pCT analyses are summarised in Table 23. Axial sections of a pCT-derived BF parameter map from baseline and the end of the second week of CRT can be seen in Figure 56. Median BF was 28.9 mL/100 g/min pre-CRT and 64.4 mL/100 g/min at the end of the second week of CRT. An increase in BF of greater than 20% was observed in 3 of the 5 patients. In the remaining 2 patients, the percentage change in BF was less than 10%. Median BV was 3.5 mL/100 g at baseline and 4.5 mL/100 g at week 2. An increase in BV of greater than 20% was observed in 4 out of 5 patients. Changes in BF and BV can be seen in Figure 57. Patient A, who had an increase in FMISO uptake from baseline to week 2 did not demonstrate an increase in BF or BV.

Table 23: Perfusion CT and dce-MRI quantification of perfusion at baseline and at the end of the second week of CRT (2wk).

Patient	pCT						dce-MRI		
	Blood flow (mL/100 g/min)			Blood volume (mL/100 g)			K _{trans} (min ⁻¹)		
	Baseline	2 wk	%Δ	Baseline	2 wk	%Δ	Baseline	2 wk	%Δ
A	22.2	20.9	-5.9	2.3	2.3	0.0		0.310	
B	38.2	68.6	68.6	3.5	4.5	28.6	0.134	0.192	44.0
C	21.5	45.1	45.1	3.2	4.1	28.1	0.186	0.414	123.1
D	28.9	136.7	136.7	4.8	6.4	33.3	0.411	0.499	21.5
E	61.6	6.8	6.8	6.2	9.1	46.8	0.676	0.764	13.1

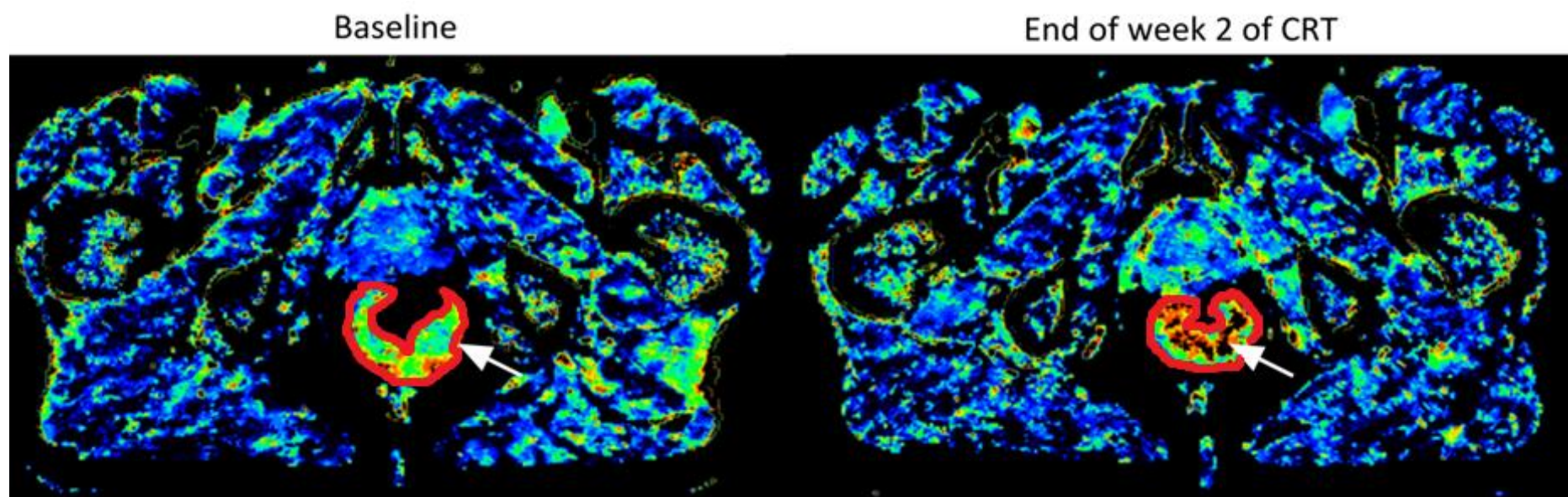


Figure 56: Axial sections from a BF parameter map from pCTs taken at baseline and at the end of the second week of CRT. The tumour (indicated) had BF and BV characterised from a ROI (red line).

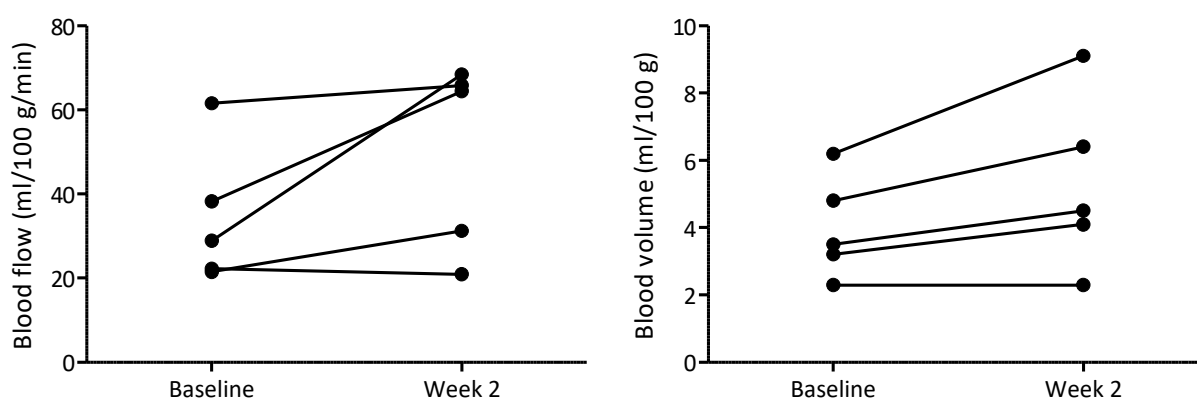


Figure 57: Changes in pCT-defined BF and BV from baseline to the end of the second week of CRT.

5.3.3.5 Dynamic contrast-enhanced MRI

Dynamic contrast-enhanced MRI scans were obtained in all 5 of the patients at baseline and at the end of the second week of CRT. The pre-CRT dce-MRI data from patient A was corrupted within the MRI scanner software and therefore could not be analysed. The scan at 2 weeks post CRT in this patient could be analysed. K_{trans} values, a measure of diffusion of tracer from the circulation to the interstitial space, are summarised in Table 23 and displayed in Figure 58. Median K_{trans} was 0.300 s^{-1} and 0.457 s^{-1} at baseline and 2 weeks respectively in the 4 patients with paired imaging. A $>20\%$ increase in K_{trans} was observed in 3 of the 4 patients.

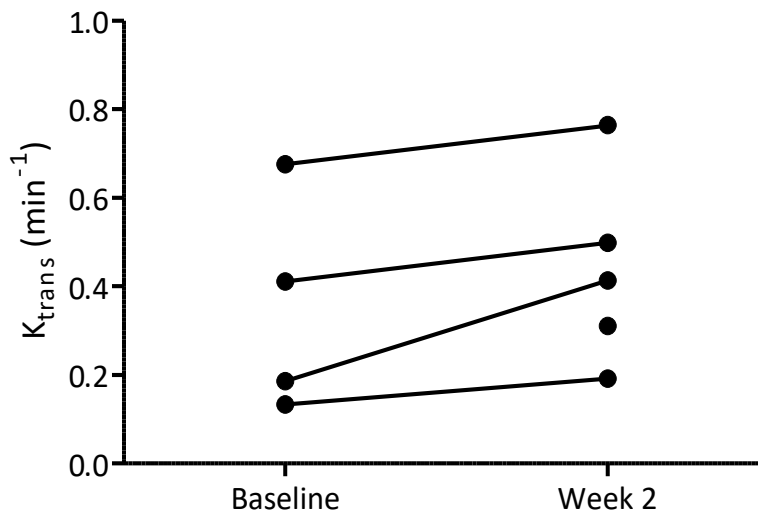


Figure 58: Changes in dce-MRI-derived K_{trans}

5.3.3.6 CD31 immunohistochemistry

Results of the quantification of CD31 staining of rectal cancer biopsy specimens can be found in Table 24. An example of a 20x magnification image that was used to perform Chalkley counts can be seen in Figure 59. The Chalkley counts decreased in 3 of the 5 patients and were unchanged in the remaining 2. The percentage of the biopsy staining positive for CD31 varied widely between patients. The CD31 positive area ranged from 2.9 to 63.4% of the biopsy section area pre-CRT and 4.4 to 82.1% at 2 weeks. The CD31 staining was reported to be increased in 3 patients using the automated method with a <10 % change in the remaining 2 patients.

Table 24: Quantification of CD31 staining of rectal cancer biopsies at baseline and at the end of the second week of CRT.

Patient	CD31						
	Chalkley Count			Quantitative (% of biopsy positive)			
	Baseline	2 wk	Δ	Baseline	2 wk	% Δ	Δ
A	7	6	↓	63.4	67.1	5.8	↔
B	4	4	↔	2.9	4.4	51.7	↑
C	5	5	↔	9.2	8.3	-9.8	↔
D	8	5	↓	8.5	74.8	780.0	↑
E	8	3	↓	13.3	82.1	517.3	↑

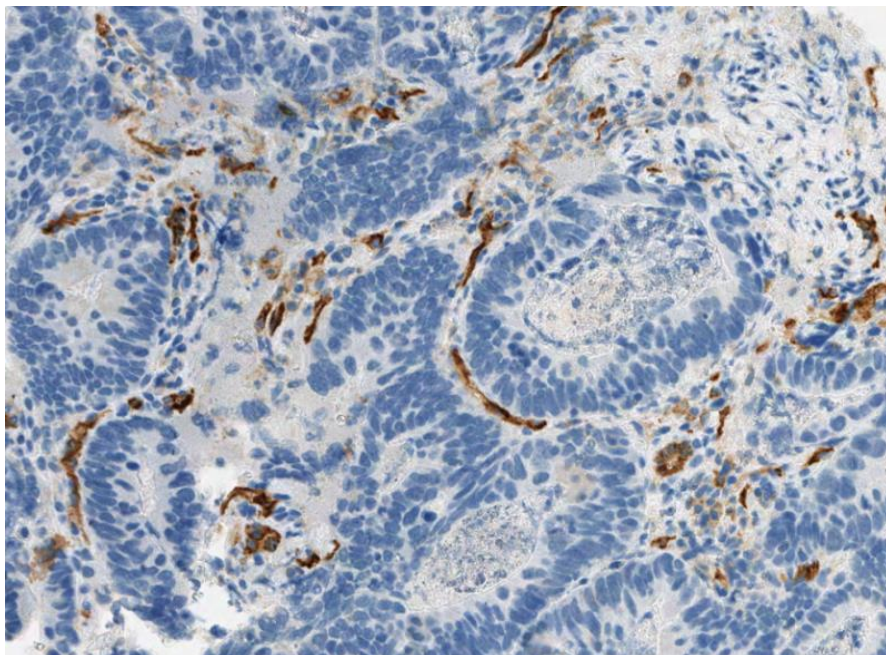


Figure 59: DAB staining of a rectal cancer biopsy for CD31 viewed at 20x magnification for Chalkley counts to quantify microvessel density.

5.3.3.7 Assessment of the response to chemoradiotherapy

One of the 5 patients did not go on to TME post CRT (patient E). This decision was made in part on patient preference, but also because of post-CRT restaging investigations suggesting pCR. At the time of writing, this patient still has a complete clinical response. H&E sections from the four resected rectal tumours can be seen in Figure 60. Of the four resected tumours, 2 patients had Mandard TRG 3 and 2 had Mandard TRG 4. Two of the resected tumours

showed a mucinous morphology. Radiological responses varied from TRG 1 to 5. The radiological response score was not in agreement with Mandard TRG. The radiological and pathological regression grades can be seen in Table 25.

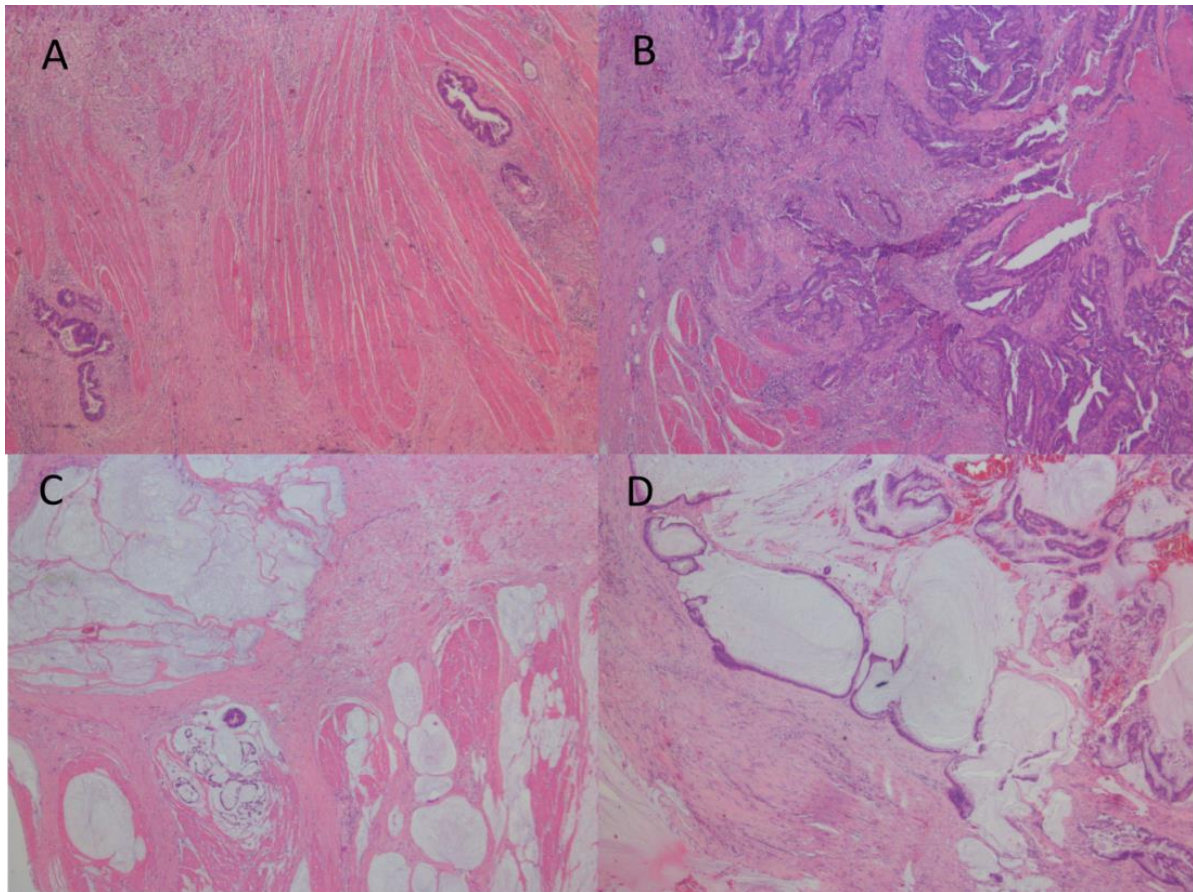


Figure 60: H&E-stained section of the resected rectal tumours. Panels A and C show responses consistent with Mandard TRG 3. B and D show sections consistent with Mandard TRG 4. Different morphologies can be seen with A and B showing residual rectal adenocarcinoma and C and D showing mucinous adenocarcinomas.

Table 25: Radiological and pathological response to CRT in the first 5 patients recruited to RHYTHM Cohort B.

Patient	Radiological TRG	Mandard TRG
A	5	3
B	2	3
C	4	4
D	2	4
E	1	N/A

5.3.3.8 *RNA extraction from biopsy material*

It was possible to extract RNA from all of the tumour biopsies that had confirmed invasive adenocarcinoma. The quality of the RNA has been tested. RNA concentrations ranged from 111 to 452 ng/ μ L. All samples have been successfully sequenced.

5.4 Discussion

The RHYTHM study has been successfully developed, taken through the trials regulatory process and opened to recruitment. While the number of patients recruited so far has been small, the trial is still open and actively recruiting. No patients approached to participate in the study declined entry because of the intensity of the imaging protocol and no patients withdrew from study participation once recruited. This reassures that the trial protocol is acceptable to patients.

The trial opened to recruitment in December 2013. Recruitment to the trial has been slower than predicted. Recruitment to RHYTHM A has been low because of the speed at which patients are offered surgical resection, mostly less than 10 days from the time of MDT discussion. This did not give time for the patient to consider trial participation, consent, and the additional biopsy and imaging tests prior to the operating date. A protocol amendment has been approved by the REC that means that patients can now be approached and given a patient information leaflet as soon as a diagnosis of rectal cancer is being considered rather than waiting for histological and imaging confirmation. There was also a period in which the FMISO tracer was not available. This is because of the suspicion of bacterial contamination of the tracer when used in another clinical study. This led to the prolonged shutdown of the external laboratory that supplied the FMISO until the quality and sterility of its tracer

production could be assured and passed as acceptable by the Medicines and Healthcare products Regulatory Agency. This substantially delayed study recruitment.

Cohort A of the RHYTHM-I study will continue to generate images that quantify hypoxia and perfusion, produce parameter maps of the spatial distribution of hypoxia and perfusion that can be then compared with the distribution of pimonidazole staining on pathology sections that have been taken in a way that characterises hypoxia across the whole tumour. Collaborative work with the department of Biomedical Image Engineering in Oxford to register IHC-stained section images with *in vivo* imaging is underway. The resected rectum sample handling and imaging pathway has been tested, proven to be feasible and generates images that aid accurate image registration. While only one patient has been recruited to Cohort A, the pCT-derived BF and BV and the test-retest variability (16.4% and 23.4% respectively) in this patient were comparable with those seen in published series.²⁷² This degree of variability compares favourably with that seen in other tumour sites. BF and BV parameter values have been seen to vary from 10.9 to 114.4% and 25.3 to 117.6% respectively in patients when a similar method to the one used here was used in patients with lung cancer.²⁷³ In this series, Ng *et al.* attributed the large variation in parameter values to tumour movement during breathing motion. In liver tumours, the test-retest variability in BF and BV has been shown to be 11.2% and 14.4% respectively when motion correction was applied to the pCT data.²³²

While it has not been possible to assess the test-retest variability of FMISO-PET or to compare regions of FMISO uptake with pimonidazole staining of tumour sections in the first patient recruited to Cohort A of the RHYTHM-I study, some modifications in the way the FMISO-PET images are acquired and interpreted may increase the likelihood of acquiring an interpretable

image in future patients. Unfortunately, both FMISO-PET/CTs in the only patient recruited to RHYTHM Cohort A demonstrated tracer signal from the rectal lumen. Spill-over of the rectal luminal tracer activity, and from the bladder, made quantification of tumour FMISO retention 4 h post injection impossible. This was particularly disappointing in this patient as there were consistently high levels of pimonidazole staining throughout the resected rectal tumour sections. High levels of rectal luminal FMISO were also seen in 3 of the 10 FMISO-PET/CTs in RHYTHM B. Although some colonic excretion of FMISO was expected,²⁷⁴ because of liver metabolism and intestinal excretion of tracer metabolites, the extent to which the FMISO would reach the rectum by 4 h post injection could not have been anticipated from published series. A protocol amendment has been approved by the ethics committee to allow all patients recruited to the study in future to have a micro-enema just prior to the 4 h post-injection scanning time-point. Analysis of the dynamic data has begun. It could be that differences in the FMISO TAC in the tumour ROI and rectal lumen in the early part of the scan can be exploited to characterise regions of hypoxia within the rectal tumour without relying on the 4 h PET image.

Because the 4 h post-tracer injection image is complicated by luminal FMISO signal, an assessment of the usefulness of FMISO-PET in characterising changes in hypoxia from baseline to the end of the second week of CRT was only possible in 3 of the 5 patients recruited so far to RHYTHM-I Cohort B. So far, the results have been promising. Visual inspection of the region of the SUV_{peak} and $T:M > 1.3$ and $T:B > 1.4$ on the two scans shows that the region of FMISO uptake is geographically stable within the TV. This suggests that FMISO-PET-derived hypoxic volumes may be a good candidate for selective radiotherapy dose escalation. The degree of overlap between the regions of FMISO uptake needs to be quantified when more images have

been acquired. In the 3 patients with interpretable, paired FMISO-PET imaging, the degree of hypoxia, whether assessed by FMISO uptake volume or maximum uptake values, was seen to decrease in 2 patients and increase in 1 patient. This supports the hypothesis that there is heterogeneity in the rate of tumour reoxygenation during fractionated CRT and, with the accrual of more patients in the study and the modifications to the FMISO-PET acquisition as described, this heterogeneity in response may be predictive of CRT outcomes.

It was thought that changes in tumour hypoxia during fractionated CRT may be the result of changes in tumour perfusion. It was possible to quantify BF and BV using the pCT method adopted in the study. An additional measure of tumour perfusion, K_{trans} from dce-MRI, could also be quantified in all but one of the dce-MRI studies. The unsuccessful dce-MRI was the result of a problem with data transfer from the MRI scanner in the first patient recruited to the study. This problem was resolved and did not recur. Perfusion CT-derived BF was seen to increase in 3 of the patients from baseline to the end of the second week of CRT, while a <10% change was seen in 2 patients. A >20% increase in tumour BV was seen in 4 of the 5 patients. K_{trans} was seen to increase in all 4 of the evaluable patients. Interestingly, of the 3 patients with evaluable, paired FMISO-PET imaging, the 2 patients who demonstrated a marked reduction in FMISO retention also demonstrated an increase in BF, BV and K_{trans} . The patient who demonstrated an increase in tumour hypoxia did not show a demonstrable increase in tumour BF or BV. These early findings are encouraging and support the hypothesis that reoxygenation during CRT is a result of increased perfusion, presumably as a result of neovascularisation and not simply the result of a reduced oxygen consumption as a result of cancer cell death. With further patient recruitment, a correlation between persistent or increasing FMISO retention and a failure to increase tumour BF may be seen.

A greater than 10% change in the serum level of OPN was observed from pre-CRT to reassessment following 10 fractions of CRT. While serum OPN level has been shown to be prognostic in a number of tumour sites,^{121,275-277} its use in rectal cancer has not been reported, nor has the change in OPN during CRT been used as a surrogate for hypoxia modulation during fractionated CRT. In the 3 patients with paired FMISO-PET imaging, the direction of change in FMISO retention or serum OPN were in agreement. Further data are required to assess a correlation between FMISO-PET and OPN-reported hypoxia modulation, but these early results are promising.

Results of the CAIX and CD31 staining of the tumour biopsies taken in patients recruited to RHYTHM B reflect tumour heterogeneity and are therefore difficult to interpret. The direction of change in CAIX-reported hypoxia, using the validated semi-quantitative method or the automated quantification of CAIX staining, did not agree with the changes in FMISO uptake in the 3 patients with evaluable paired imaging. It can be seen in Figure 54 that the areas of FMISO uptake are not seen at the endoluminal surface and are not seen the full length of the TV. It is therefore perhaps unsurprising that reporting hypoxia using a small, surface biopsy offers different results. Interestingly, all tumour biopsy sections had areas of CAIX positivity. A greater understanding of microscopic variations in hypoxia and how they relate to FMISO uptake on a voxel-by-voxel basis is required. Chalkley Counts of CD31-stained biopsy sections decreased from baseline to the end of week 2 of CRT despite imaging measures of perfusion increasing in 4 out of 5 patients. Some agreement was seen using the automated quantification of CD31 positivity where CD31 staining was shown to increase in the 4 patients who demonstrated an increase in tumour perfusion and remain unchanged in the one patient who did not show an increase in pCT-derived BF. This correlation suggests that this method

of characterising changes in tumour perfusion shows promise and should be assessed further in patients who are recruited to the RHYTHM study in future. It also suggests that changes in perfusion can be seen in endoluminal surface biopsies and may reflect geographical variation in tumour reperfusion in tumours, with the outer edge of the tumour being most important in neovascularisation in response to radiation. This hypothesis could be tested in future patients.

Invasive tumour was sampled at each time-point in all of the patients. The method adopted here of bisecting each biopsy and formal fixing and paraffin embedding one half of each biopsy for diagnostic confirmatory purposes was successful and allowed selection of only biopsies that contained invasive adenocarcinoma to be used for RNA sequencing. Storing half of the bisected biopsy in RNeasy[®] allows for the extraction of good quality RNA that was successfully sequenced. The results of the RNA sequencing are currently being analysed. These will be used to generate a rectal cancer-specific hypoxia metagene panel as well as comparing the gene expression information with hypoxia metagene panels derived from other tumour types in collaboration with Professor Catharine West (Institute of Cancer Science, University of Manchester). Differences in CAIX staining of biopsies and hypoxia reported with FMISO-PET further highlight the challenges in using a small, endoluminal biopsy as a surrogate for the biology of the whole tumour. Inevitable tumour heterogeneity and the inadequacy of a single biopsy to describe this heterogeneity must be taken into account when interpreting the results of the RNA sequencing of the tumour biopsy material.

One of the central aims of the RHYTHM study, Cohort B, is to see if changes in hypoxia and perfusion as described by FMISO-PET, pCT and dce-MRI can be used to predict pCR. Unfortunately, of the 5 patients recruited to Cohort B to date, no pCR (or Mandard TRG 1) has

been seen. This is not unusual as fewer than 30% of patients achieve pCR post CRT.³⁶ A range of Mandard TRGs, from 1 to 5, will need to be seen to get a feeling of how useful the imaging methods employed here are at predicting pCR or failure of response. It may be that the number of patients recruited to RHYTHM-I needs to be expanded from the 10 patients currently expected in Cohort B. This should be revisited when the first 10 patients have been recruited.

5.5 Conclusions

The RHYTHM clinical study is open to recruitment and the imaging and biopsy schedule is acceptable to patients. The method of handling and imaging the resected rectal specimen in patients recruited to Cohort A of the study, with proposed changes to the FMISO-PET acquisition protocol, should allow the comparison of FMISO-PET-derived hypoxic volumes with whole-mount histology of pimonidazole in staining across the whole TV. Cohort A will also allow the test-retest variability of FMISO-PET and pCT to be quantified. Progress has already been made in producing software that will allow the accurate registration of tumour histopathology sections with *in vivo* imaging. Interpretation of FMISO-PET images of rectal tumour is complicated by colonic excretion of FMISO with FMISO seen within the rectal lumen by 4 h post injection. Visual assessment of sequential FMISO-PETs in the 3 patients recruited to Cohort B who had interpretable imaging suggests that the area of FMISO uptake is geographically stable and therefore shows promise as a means of delineating a BTV for selective radiotherapy dose escalation. Heterogeneity in changes in hypoxia and perfusion imaging could be seen. With more patients recruited to this trial protocol, a correlation between FMISO-PET or perfusion imaging with histopathological response may be observed. It was possible to detect a change in circulating OPN during CRT in all of the patients and, where possible, the change appears to correlate with FMISO-PET-reported changes in

hypoxia. It was possible to obtain tissue containing invasive adenocarcinoma in all patients at each time-point. The failure of a superficial biopsy to describe intratumoural heterogeneity may limit their application as a surrogate for more detailed imaging characterisation of tumour biology. The full complement of patients need to be recruited before the utility of FMISO-PET, pCR and dce-MRI in predicting pCR can be assessed and before the null hypotheses as outlined in section 5.2 are accepted or rejected.

Chapter 6: Conclusions and future directions

Treatment outcomes in LAPC are poor. Only a minority of patients with LARC who receive preoperative CRT have a sustained response. There is a need to improve responses to CRT in both of these patient groups. An important first step in developing methods to improve CRT is to accurately identify patients who are likely to have a suboptimal response to treatment and may therefore benefit from local treatment intensification.

From my review of the literature, I developed the hypothesis that studies that used PET-derived parameters in response to predict response to CRT may be improved if the SUV_{max} , which is subject to noise, was replaced with a more detailed assessment of PET tracer uptake. In fact, my observation in LAPC was that the SUV_{max} correlates very closely with other FDG-PET parameters and therefore can be used as a good summary of the tumour glucose uptake. This is advantageous as the SUV_{max} is the most easily derived PET parameter and is often included in routine clinical imaging reports. This may make future data collection more efficient and adds confidence to the continued use of SUV_{max} .

My observation that low FDG uptake in the tumours of patients with LAPC was associated with a failure of the tumour to spread distant sites needs to be validated in a larger cohort of patients. A robust means of identifying this group of patients may identify those who may derive clinical benefit from local treatment intensification. A Phase II clinical trial (RTOG 1201) is currently investigating the use of SMAD4 as means of identifying a group of patients with LAPC who may benefit from radiotherapy dose escalation. FDG-PET may prove to be another means of identifying this patient group. Using FDG-PET to inform this treatment strategy decision warrants further investigation as obtaining pancreatic tissue for SMAD4

characterisation can be challenging. In terms of the prognostic utility of FDG-PET in LAPC, an improvement in OS was observed in patients in whom the decrease in SUV_{max} from pre- to post CRT was greater than the median for the group. This observation further supports the ongoing investigation of FDG-PET in guiding treatment strategy selection in LAPC. Large numbers of patients will be needed to validate any FDG-PET-derived parameter that could be used to inform treatment decisions. This will require robust quality assurance of standardised imaging protocols and a multicentre collaborative approach.

Areas of FDG avidity in LAPC pre-CRT identified areas of residual metabolic activity post CRT. While these data may be used to support the use of FDG-PET in deriving a BTV for radiotherapy dose escalation, it must be remembered that the imaging analysis was only possible in approximately 50% of patients. It should also be remembered that there is not, as yet, a known correlation between patterns of post-CRT FDG avidity and modes of disease progression. FMISO-PET shows promise as a means of defining a BTV in LAPC. All of the pancreatic tumours imaged here demonstrated areas of FMISO retention, which appeared to be geographically stable on repeat testing. This is the first report of sequential FMISO-PET studies demonstrating reproducible tracer uptake in LAPC. As FMISO informs about hypoxia, and hypoxia is a well-documented cause of chemo- and radioresistance, its utility in BTV definition in LAPC requires further investigation. The dynamic FMISO-PET studies and pCT performed in a small number of patients receiving nelfinavir monotherapy suggested that nelfinavir may have a role in hypoxia modification, perhaps by increasing tumour perfusion. A Phase I/randomised Phase II study in LAPC will open to recruitment in 2016 (SCALOP-2; NCT02024009). This study will randomise patients between CRT and CRT plus nelfinavir. The SCALOP-2 study would be a perfect vehicle to test this hypothesis further. A baseline

assessment of tumour hypoxia in all patients, with repeat assessment during CRT, would allow changes in hypoxia and perfusion during CRT to be quantified in patients receiving CRT with and without nelfinavir. This may provide convincing evidence that nelfinavir can improve tumour oxygenation during CRT.

A reliable means of extracting information from routine anatomical imaging that can be successfully used in CRT response prediction would reduce the imaging burden to patients and healthcare providers. A software tool to characterise texture features of clinical images was developed. Pre- and post-CRT CECT images from patients with LARC were analysed. The imaging data set used to try to identify a texture 'signature' that predicts response was small, with little diversity in treatment outcomes, and the imaging protocol used to acquire the CT images was not standardised in each patient. This analysis could be improved by prospective data collection, standardisation of the CT imaging protocol and pre-processing of the CT images with reduced noise and more accurate texture maps. The image analysis software tool may then identify a number of texture features that predict CRT response.

The RHYTHM study aims to develop a robust means of characterising hypoxia and perfusion in rectal tumours, including the quantification of change in hypoxia and perfusion during preoperative CRT. The study is still open to recruitment. The early experience of the study presented in Chapter 5 of this thesis suggests that the imaging and biopsy schedule is acceptable to patients. A protocol amendment to allow the administration of a micro-enema prior to the imaging 4 h after FMISO injection has already been approved. The impact that this step has on luminal tracer signal needs to be assessed. It may be difficult to support further use of FMISO-PET to characterise rectal tumour hypoxia if a significant proportion of scans are uninterpretable because of PET tracer in the rectal lumen. Work has already begun

to see if the data from the dynamic FMISO scans can be used to characterise rectal tumour hypoxia. By analysing the FMISO kinetics on a voxel-by-voxel basis, it may be possible identify tumour FMISO retention rather than rectal luminal activity. Early results of the pharmacokinetics of FMISO in the rectal tumour and lumen show promise as they appear to be sufficiently different that true tumour FMISO retention may be distinguishable from luminal tracer excretion (see Figure 61, courtesy of Jenny Gotwald).

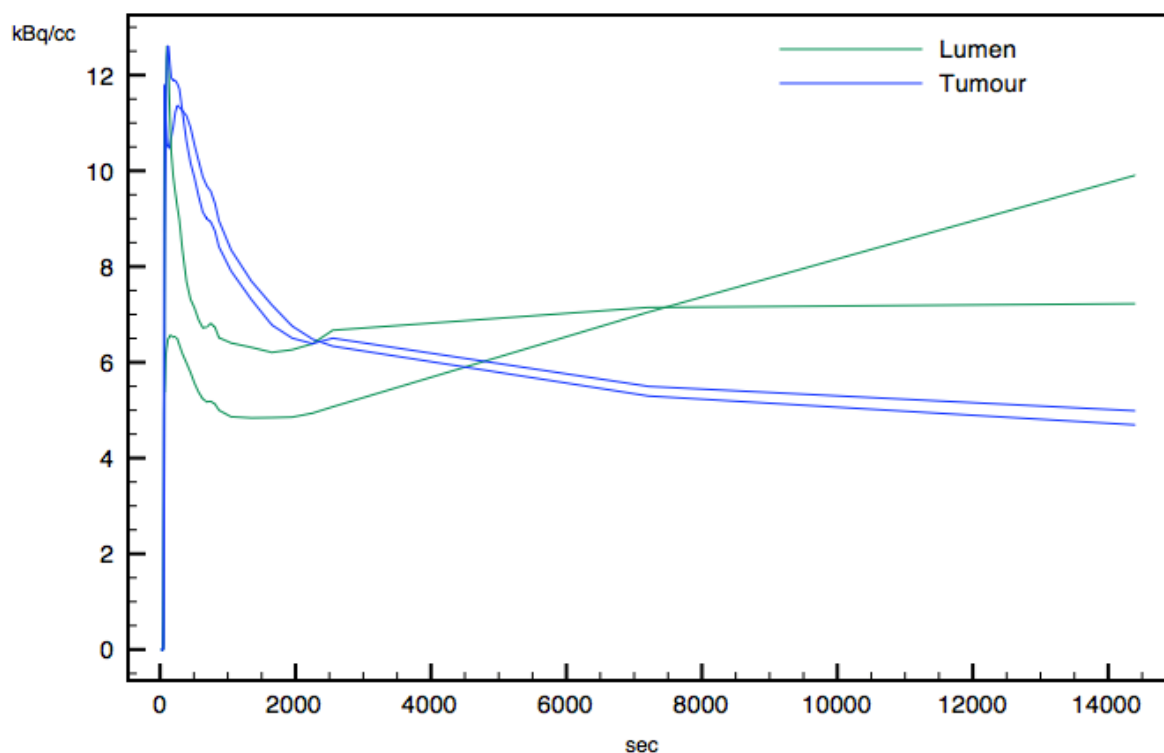


Figure 61: Difference in FMISO pharmacokinetics in rectal tumours (blue) and the rectal lumen (green) in two patients.

Further work is required to develop the registration software that will allow comparison of clinical imaging with histological tumour sections. A method of detecting pimonidazole in the rectal tumour has been established. Correlation of the spatial distribution of pimonidazole staining with FMISO-PET images may further increase confidence in the use of FMISO-PET to

define hypoxia in human tumours and may provide a basis on which to segment the PET image to generate an accurate representation of tumour hypoxia.

The RHYTHM trial needs to complete recruitment before an assessment of the utility of a multimodality assessment of hypoxia and perfusion in predicting response to CRT can be made. Once all of the imaging has been acquired and correlated with histological markers of hypoxia and perfusion, a method of accurately defining a BTV for radiotherapy dose escalation may emerge. Radiotherapy dose escalation to a hypoxic BTV, derived from either FMISO-PET or an accurate representation of areas of poor perfusion from the dce-MRI imaging, may eventually improve CRT outcomes for patients with LARC.

Functional imaging in both LAPC and LARC shows promise as a means of personalising CRT – either by aiding treatment strategy selection or by directing local treatment intensification.

Appendix A

Literature search strategies

The following four searches were performed in 'Ovid MEDLINE ® In-Process & Other Non-Indexed Citations and Ovid MEDLINE ® 1946' on 15th of January 2015:

- **Search A**

1. Pancreatic Neoplasms/
2. ((pancreas or pancreatic) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
3. 1 or 2
4. exp magnetic resonance imaging/ or radionuclide imaging/ or perfusion imaging/ or exp tomography, emission-computed/
5. exp Tomography, X-Ray Computed/
6. 4 or 5
7. exp Prognosis/
8. 3 and 6 and 7
9. limit 8 to english language

- **Search B**

1. Pancreatic Neoplasms/
2. ((pancreas or pancreatic) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
3. 1 or 2
4. exp magnetic resonance imaging/ or radionuclide imaging/ or perfusion imaging/ or exp tomography, emission-computed/
5. exp Tomography, X-Ray Computed/
6. 4 or 5
7. Radiotherapy, Image-Guided/
8. Radiotherapy Planning, Computer-Assisted/
9. 7 or 8
10. 3 and 6 and 9
11. Target definition.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
12. Target volume.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
13. (chemoradiotherapy or radiotherapy).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
14. 11 or 12
15. 13 and 14

16. 3 and 6 and 15
17. limit 16 to english language

- **Search C**

1. Rectal Neoplasms/
2. ((rectal or rectum) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
3. 1 or 2
4. exp magnetic resonance imaging/ or radionuclide imaging/ or perfusion imaging/ or exp tomography, emission-computed/
5. exp Tomography, X-Ray Computed/
6. 4 or 5
7. exp Prognosis/
8. 3 and 6 and 7
9. limit 8 to english language

- **Search D**

1. Rectal Neoplasms/
2. ((rectal or rectum) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
3. 1 or 2
4. exp magnetic resonance imaging/ or radionuclide imaging/ or perfusion imaging/ or exp tomography, emission-computed/
5. exp Tomography, X-Ray Computed/
6. 4 or 5
7. Radiotherapy, Image-Guided/
8. Radiotherapy Planning, Computer-Assisted/
9. 7 or 8
10. 3 and 6 and 9
11. Target definition.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
12. Target volume.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
13. (chemoradiotherapy or radiotherapy).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
14. 11 or 12
15. 13 and 14
16. 3 and 6 and 15
17. limit 16 to english language

The following four searches were then performed in Embase 1974 to 2015 Week 2:

- **Search E**

1. exp pancreatic cancer/

2. ((pancreas or pancreatic) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
3. 1 or 2
4. exp functional magnetic resonance imaging/ or exp nuclear magnetic resonance imaging/ or exp positron emission tomography/
5. exp treatment outcome/
6. 3 and 4 and 5
7. limit 6 to english language

- **Search F**

1. exp pancreatic cancer/
2. ((pancreas or pancreatic) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
3. 1 or 2
4. exp functional magnetic resonance imaging/ or exp nuclear magnetic resonance imaging/ or exp positron emission tomography/
5. exp computer assisted radiotherapy/ or exp treatment planning/
6. exp multimodality cancer therapy/
7. 5 and 9
8. 3 and 4 and 7
9. limit 8 to english language

- **Search G**

1. exp rectum cancer/
2. ((rectal or rectum) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
3. 1 or 2
4. exp functional magnetic resonance imaging/ or exp nuclear magnetic resonance imaging/ or exp positron emission tomography/
5. exp treatment outcome/
6. 3 and 4 and 5
7. limit 6 to english language

- **Search H**

1. exp rectum cancer/
2. ((rectal or rectum) adj2 (cancer or adenocarcinoma* or carcinoma*)).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
3. 1 or 2
4. exp functional magnetic resonance imaging/ or exp nuclear magnetic resonance imaging/ or exp positron emission tomography/
5. exp computer assisted radiotherapy/ or exp treatment planning/
6. exp multimodality cancer therapy/
7. 5 and 9
8. 3 and 4 and 7
9. limit 8 to english language

Paper selection

Original articles in English reporting studies including the search terms functional imaging, CRT and rectal cancer or pancreatic cancer were listed. In cohorts receiving radiotherapy alone or CRT as neoadjuvant therapy, the study was only included if separate data for those receiving CRT was supplied. Small series (<20 patients) that were unable to demonstrate a statistical significance between groups were excluded unless a description of how the cohort size was selected to ensure sufficient statistical power to meet its end-point was included. Papers that provided insufficient details on their approach to image analysis or the statistical methods employed were excluded.

Table I: The predictive and prognostic utility of functional imaging in pancreatic cancer. All studies used FDG-PET.

	Ref.	n.	Total tumour radiation dose and chemotherapy agents	Definition of response	Imaging parameter	Sens, Spec PPV, NPV (%)	Comments
Predictive	55	40	50 Gy in 25 # Gemcitabine	<50% viable tumour cells post CRT	Pre-SUV _{max} ≥ 4.7 Decrease SUV _{max} > 46% (median) Meets both cut-offs	67, 84 -, -	71% 'response' vs 32% (p = 0.03) 71% vs 26% (p = 0.01) FDG-PET pre-CRT and 8 weeks post CRT
Prognostic				Survival end-point			
	278	NR	50.4 Gy in 28 # or 50 Gy in 40 # Gemcitabine	OS	Pre-SUV _{max} < 7.0		Improved median OS (magnitude NR) (p < 0.05)
	279	15	NR	TTP	SUV _{max} reduction >50%		Mean TTP 399 vs 233 days (p < 0.05)
	236	32	50.4 Gy in 28# 5-FU	OS	SUV _{max} reduction > 63.7%		Median OS 17.0 vs 9.8 months (p = 0.009) Median LRPFS 12.3 vs 6.9 months (p = 0.02)
	280	30	50.4 Gy in 28 # 5-FU	OS	FDG-PET CT-derived GTV < 91.1 cm ³		Median OS 14.1 vs 9.5 months (p = 0.005)
	44	260	50.4 Gy in 28 # (median dose)	OS 12-month OS	Pre-SUV _{max} < 3.5 SUV _{max} reduction > 60% Pre-SUV _{max} < 3.5 SUV _{max} reduction > 60%	83, 42 20, 93 92, 23 23, 92	Median OS 23.0 vs 13.2 months Median OS 41.9 vs 16.0 months

Table II: Using functional imaging prior to CRT to predict treatment response in LARC.

Ref	n	Imaging modality	Tumour radiation dose and chemotherapy agents	Response to be predicted	Imaging parameter	Sens, Spec PPV, NPV	ROC curve AUC	Comments
²⁸¹	17	dce-MRI	38.3-45 Gy in twice daily 1.1 Gy # Infusional 5-FU on days of RT	T downstaging	<15% of pixels with perfusion index >12.6 mL/min/100 g	100, 100 -, -		High PI associated with reduced likelihood of T downstaging
⁶²	34	dce-MRI and dw-MRI	38.3-45 Gy in twice daily 1.1 Gy # Infusional 5-FU on days of RT	T downstaging	<12% of pixels with a PI >12 mL/min/100 g	All but one case correctly identified		Baseline mean ADC no different in responders and non-responders, but higher relative fraction of ADCs in non-responders Responders had a lower mean PI (p < 0.001)
²⁸²	19	pCT	50.4 Gy in 28 # Capecitabine	T or N downstaging				BF, BV, mean transit time all significantly higher in 'responders'
²⁸³	58	dce-MRI	45 Gy in 25 # 5-FU	T downstaging	<15% of pixels with PI >12.6 mL/min/100 g	83, 100 100, -		Higher PI in non-responders than responders (p < 0.0001)
⁴⁷	49	dw-MRI	45–50.4 Gy over mean of 6 weeks 5-FU +/- oxaliplatin or capecitabine	T downstaging	ADC > 0.833 x 10 ⁻³ Reader 1 Reader 2	75, 48 76, 48 65, 52 75, 40	0.676 0.634	ADC higher in responders than non-responders (p = 0.035)
²⁸⁴	81	FDG-PET	45 Gy in 25 # 5-FU	pCR	SUV _{max} , SUV _{mean} , metabolic TV, TLG			No difference between responders and non-responders
²⁸⁵	38	FDG-PET	Induction 5-FU + oxaliplatin 45-50.4 Gy in 25-28 # Tegafur 10–12.5 Gy intraoperatively	Dworak TRG 3-4	SUV _{max} ≥ 8.4 (median) SUV _{max} ≥ 8.4 and overexpression of VEGFR-2	79, 79 79, 79 89, 79 81, 88		

Table III: Using imaging during CRT in addition to baseline and post-treatment imaging to predict treatment outcome in LARC

Ref	n	Imaging modality	Tumour radiation dose and chemotherapy agents	Response to be predicted	Imaging parameter	Sens, Spec PPV, NPV (%)	ROC curve AUC	Comments
²⁸⁶	8	dw-MRI	38.3-45 Gy in twice daily 1.1 Gy Infusional 5-FU on days of RT	T downstaging				Weekly MRI. 2/4 'responders' showed an increase in mean ADC at week 1 of CRT cf. 0/4 'non-responders' (p < 0.01)
⁶⁸	33	FDG-PET	45 Gy in 25 # Oxaliplatin, raltitrexed, 5-FU	Mandard TRG1-2	% Δ SUV _{max} $\geq$ 42% at day 12 % Δ SUV _{mean} $\geq$ 42% at day 12	100, 87 -, - 100, 100 100, 100	0.950 1.000	Imaging at baseline and day 12 Median Δ SUV _{max} from baseline to day 12 in 'responders' vs 'non-responders' 62% vs 28% (p < 0.0001) Post-CRT or overall change in SUVs did not correlate with treatment outcome
⁴⁹	10	FLT-PET	45 Gy in 25 # 5-FU	<10% residual tumour cells	Absolute SUV _{mean} , SUV _{max} , % Δ SUV _{max} , % Δ SUV _{mean}			Imaging at baseline, 2 weeks and post CRT SUV _{mean} decreased at each time-point (p = 0.005). No difference in any of the reported parameters between responders and non-responders
²⁸⁷	31	FDG-PET	50.4 Gy in 28 # Capecitabine or 5-FU +/- oxaliplatin	Mandard TRG 1-2	SUV _{max} during CRT < 7.3 Post-SUV _{max} < 4.4 % Δ SUV _{max} > 49.1 during CRT % Δ SUV _{max} > 60.0 post CRT	79, 78 88, 64 77, 89 94, 62 63, 56 75, 42 77, 56 81, 50	0.825 0.886 0.673 0.667	Imaging at baseline, 'within 3 weeks' of starting CRT (28/31 patients) and 12 weeks post CRT

288	29	FDG-PET	45 Gy in 25 # 5-FU	Becker TRG 1 ($<10\%$ viable tumour cells)	$\% \Delta \text{SUV}_{\text{max}} \geq 35$ at Day 14	74, 70 82, 58	0.700	
					$\% \Delta \text{SUV}_{\text{max}} \geq 57.5$ post CRT	79, 70 83, 64	0.737	
289	30	FDG-PET	50.4 Gy in 28# Capecitabine	Mandard TRG 1-2	$\% \Delta \text{SUV}_{\text{max}}$ at day 8	N/A	N/A	No separating threshold found to separate 'responders' from 'non- responders' at day 8 By excluding patients with FDG uptake consistent with inflammatory changes, ROC curve AUCs improved to 0.970 and 0.890 respectively
				T downstaging	$\% \Delta \text{SUV}_{\text{max}} \geq 43$ at day 15	77, 93 91, 82	0.870	
					$\% \Delta \text{SUV}_{\text{max}} \geq 43$ at day 15	57, 85 80, 65	0.840	
48	22	FDG-PET and dw- MRI	45-50.4 Gy in 25-28 # 5-FU or 5-FU and oxaliplatin	pCR	(1) $\% \Delta \text{SUV}_{\text{max}} \geq 40$ after 10-12 #	100, 75 60, -		
					(2) $\% \Delta \text{SUV}_{\text{max}} \geq 76$ post-CRT	100, 75 60, -		
					(3) Baseline ADC $\leq$ 1.06×10^{-3}	100, 87.5 -, -		
					(1) and (2)	100, 94 86, -		
					(3) and (1)	100, 94 86, -		
					(3) and (2)	100, 100 100, 100		
287	31	FDG-PET	50.4 Gy in 28 # Capecitabine, 5-FU +/- oxaliplatin	Mandard TRG 1-2	PET2 $\text{SUV}_{\text{max}} < 7.3$	79, 78 88, 64	0.825	PET2 'within 3 weeks of starting CRT' in 28/31 patients PET3 post CRT 'within 12 weeks'
					PET3 $\text{SUV}_{\text{max}} < 4.4$	77, 89 94, 62	0.886	

					Pre-PET2 % Δ SUV _{max} > 49.1	63, 56 75, 42	0.673	
²⁹⁰	34	dw-MRI and MRI volumetry	45 Gy in 25 # 5-FU	Mandard TRG 1-2				No difference in ADC or volumetric response in responders vs non- responders at baseline, week 2 or post- CRT
²⁹¹	27	FDG-PET	50.4 Gy in 28 # 5-FU +/- cetuximab	≥95% pathological response	VRS > 50%	94, 55 -, -	0.761	FDG-PET pre- and after 8-14 days of CRT. No difference in parameters between responders and non-responders
⁶⁹	42	FDG-PET	45 Gy in 25 # Induction oxaliplatin followed by 5-FU based CRT	Mandard TRG 1-2	% Δ SUV _{mean} ≥ 52 % Δ SUV _{max} ≥ 42	100, 100 100, 100 100, 85 -, -		FDG-PET pre- and after 12 days of CRT
⁵⁶	62	dw-MRI	50.4 Gy in 28 # +/- 10 Gy boost, fractionation NR Capecitabine + oxaliplatin	Mandard TRG 4 Mandard TRG 1-2	Pre-ADC < 1.0 x 10 ⁻³ % Δ SUV _{max} > 23	83, 78 -, 95 -, - 67, 69		MRI pre-CRT, day 10-14 and post CRT
²⁹²	20	FDG-PET	50.4-54 Gy in 28-30 # 5-FU or capecitabine or UFT	pCR	% Δ SUV _{max} > 0 % Δ SUV _{max} > 32	100, 40 31, 100 75, 100 100, 100		FDG-PET pre-CRT and day 8
²⁹³	26 20	FDG-PET	50.4 Gy in 28 # Capecitabine	Mandard TRG 1-2	% Δ SUV _{max} > 48 % Δ SUV _{max} > 48	64, 100 -, - 83, 93 -, -		FDG-PET at baseline and end of week 2 of CRT Validation cohort
²⁹⁴	20	dw-MRI	45-50.4 Gy in 25-28 # 5-FU (+ oxaliplatin in 2 patients)	pCR	Pre-ADC < 1.06 x 10 ⁻³	100, 86 75, 100		Imaging at baseline, after 10-15 # and post CRT

					% Δ ADC at 10-15 # > 50 % Δ ADC pre- to post-CRT > 48	100, 100 100, 100 100, 93 86, 100		
⁷⁷	20	dce-MRI	50.4 Gy, fractionation NR 5-FU	Mandard TRG 1-2				Imaging at baseline, end of week 2, post-CRT. No difference found in absolute or relative K _{trans} values in responders and non-responders
²⁹⁵	30	FDG-PET	54 Gy in 24 # Capecitabine +/- oxaliplatin	pCR	Post-CRT SUV _{max} < 5.4	81, 100 -, 99	0.960	Imaging at baseline, after 2 weeks (24/30) and post CRT 2 week imaging not predictive
⁵⁰	13	FLT-PET and FDG- PET	45-50.4 Gy in 25-28 # 5-FU	Mandard TRG 1-3				FLT-PET at baseline and week 2 of CRT. No difference in % Δ SUV _{max} ^{FLT} between responders and non-responders
⁶⁷	28	FDG-PET	50.4 Gy in 28 # Capecitabine	Mandard TRG 3-5	% Δ SUV _{mean} > 31 at day 8 % Δ TLG > 34 at day 8 % Δ SUV _{max} > 43 at day 15 % Δ SUV _{mean} > 34 at day 15 % Δ TLG > 53 at day 15	88, 33 -, - 56, 67 -, - 88, 58 -, - 81, 67 -, - 63, 92 -, -	0.730 0.750 0.770 0.790	FDG-PET Pre-CRT, Day 8 and Day 15. Results shown are without partial volume correction which was shown to have no effect on the predictive power of the test, despite increasing the absolute SUVs

Table IV: Using imaging at baseline and post-treatment to predict CRT outcome in rectal cancer

Ref	n	Imaging modality	Tumour radiation dose and chemotherapy agents	Response to be predicted	Imaging parameter	Sens, Spec, PPV, NPV (%)	ROC curve AUC	Comments
²⁹⁶	15	FDG-PET	50.4 Gy in 28 # 5-FU	>66% response	Visual assessment, TLG, SUV _{max} , SUV _{mean}			Visual response assessment most accurately agreed with observed pathological response
²⁹⁷	16	dce-MRI	Induction 5-FU/MMC followed by 54 Gy in 30 # with 5-FU	CR/PR	ln K ^{trans}			Responders had higher K ^{trans} at baseline (p = 0.03) and larger decrease in K ^{trans} post CRT (p = 0.04)
⁵¹	15	MET-PET	45 Gy in 25 # 5-FU	Mandard TRG 1-2				No significant difference in uptake noted between responders and non-responders for pre-, post- or relative parameters
⁷⁸	20	FDG-PET	45 Gy in 25 # (plus weekly radiofrequency hyperthermia) 5-FU	CR/PR	%ΔSUV _{max} > 36.1	100, 86 93, 93	0.890	ΔSUV _{max} 59.8% 'responders' vs 29.7% (p = 0.003)
²⁹⁸	21	FDG-PET	45-50.4 Gy in 25-28 # 5-FU or tegafur-uracil	T downstaging	Mean post-CRT SUV _{max}			1.9 in those downstaged vs 3.3 (p = 0.03)
⁷⁹	23	FDG-PET	45 Gy in 25 # (plus weekly radiofrequency hyperthermia) 5-FU	CR/PR	%ΔSUV _{max} > 36.0	100, 60 77, 100	0.700	Compare with AUC of 0.515 and 0.762 for tumour shrinkage measured on CT and MRI respectively
²⁹⁹	45	FDG-PET	56 Gy in 25 # 5-FU or capecitabine	Mandard TRG 1-2	%ΔSUV _{max} > 66.2	81, 79 77, 89	0.856	ΔSUV _{max} 75.9% 'responders' vs 46.9% (p = 0.0015)
³⁰⁰	21	FDG-PET	50.4 Gy, fractionation NR Capecitabine or 5-FU +/- oxaliplatin	Microscopic disease/pCR	%ΔSUV _{max} > 75 %ΔSUV _{max} > 70	86, 85 -, - 64, 100 -, -	0.820	
³⁰¹	20	FDG-PET	50.4 Gy in 28 # Capecitabine	Mandard TRG 1-2				ΔSUV _{max} 83.0% 'responders' vs 59.4% (p = 0.025)

75	81	FDG-PET	56 Gy in 25 # 5-FU	Mandard TRG 1-2	Baseline $SUV_{max} \leq 13.8$	68, 70 70, 38	0.716	
				pCR	Post-CRT $SUV_{max} < 5.2$	81, 80 81, 80	0.794	
					Absolute $\Delta SUV_{max} \leq 6.2$	71, 88 85, 75	0.806	
					$\% \Delta SUV_{max} \leq 63.4\%$	85, 80 81, 84	0.862	
					Baseline $SUV_{max} \leq 13.9$	86, 60 81, 31	0.627	
					Post-CRT $SUV_{max} \leq 5.2$	66, 95 98, 48	0.751	
					Absolute $\Delta SUV_{max} \leq 6.4$	54, 94 94, 39	0.730	
					$\% \Delta SUV_{max} \leq 63.4\%$	51, 81 39, 81	0.786	
302	33	dce-MRI	50.4 Gy in 28 # Capecitabine, irinotecan, cetuximab	T downstaging	Change in maximum gradient of tumour TAC from pre- to post CRT			Larger degree of 'downstaging' correlated with increasing change in TAC max gradient
303	53	FDG-PET	50.4-54 Gy in 28-30# or IMRT in 2.2 Gy #s, total dose NR Capecitabine or 5-FU or 5-FU and MMC	pCR	Mean pre- and post- CRT SUV_{max} , $\% \Delta SUV_{max}$			None of the parameters reached statistical significance when comparing pCR with non- pCR
304	53	FDG-PET	45-50 Gy, fractionation NR	T or N downstaging	Absolute $\Delta SUV_{max} > 4.0$	46, 86 73, 66	0.730	

			5-FU, tegafur Or 2 cycles of 5-FU and oxaliplatin prior to tegafur-based CRT		% Δ SUV _{max} > 66	39, 82 67, 58	0.690	
³⁰⁵	20	FDG-PET	50.4-54 Gy in 28-30# 5-FU	pCR	Visual assessment			5/7 with mCR had pCR 2/10 incomplete PET response had pCR
³⁰⁶	50	FDG-PET	50.4 Gy in 28 # Capecitabine +/- oxaliplatin	Mandard TRG 1-2 pCR	Post SUV _{max} ≤ 4.24 Post SUV _{max} ≤ 4.24	70, 77 67, 79 63, 74 41, 88	0.773 0.748	Baseline and response indices not correlated with treatment outcome
³⁰⁷	37	dw-MRI	40 Gy in 20 # Capecitabine	T downstaging				Baseline ADC lower in 'responders' (p = 0.013), 'responders' show significant increase in ADC after 1 week CRT (p < 0.001)
³⁰⁸	50	dw-MRI	50.4 Gy, fractionation NR Capecitabine	pCR	Pre-ADC > 0.97 x 10 ⁻³ Post-ADC > 1.41 x 10 ⁻³ % Δ ADC < 25.3	38, 81 42, 78 46, 56 27, 74 54, 64 35, 79	0.550 0.540 0.510	No significant difference in baseline, post- CRT and change in ADC between responders and non-responders
³⁰⁹	37	FDG-PET	50.4 Gy, fractionation NR 5-FU or irinotecan + S1	Mandard TRG 1-2 pCR	Post-SUV _{max} < 4.45 % Δ SUV _{max} > 59 Post-SUV _{max} < 3.35 % Δ SUV _{max} > 75	80, 67 -, - 80, 58 -, - 85, 79 69, 91 69, 83 69, 83	0.778 0.710 0.885 0.760	

³¹⁰	76	dw-MRI	50.4 Gy in 28 # 5-FU	pCR	Pre-ADC Post-ADC > 1.30×10^{-3} %ΔADC 42% (increase)	81, 39 18, 93 100, 85 52, 100 100, 71 34, 100	0.578 0.930 0.913	
³¹¹	80	FDG-PET	50.4 Gy in 28 # 5-FU +/- oxaliplatin, 5-FU +/- panitumumab	pCR	Baseline SUV _{max} ≤ 27 Post-CRT SUV _{max} ≤ 5 %ΔSUV _{max} ≤ 66.1%	100, 11 22, 100 86, 34 25, 92		
⁷⁴	72	FDG-PET	48-50.4 Gy in 1.8-2.0 Gy #s 5-FU (+ cisplatin in 1 patient)	Dworak TRG 3-4	Post-SUV _{max} < 5.35 %ΔSUV _{max} ≥ 53.1	72, 62 -, - 70, 59 -, -	0.655 0.664	
³¹²	35	FDG-PET	50.4 Gy in 28 # 5-FU	Pathological 'near' or CR	%ΔSUV _{max} ≥ 20	40, 95 80, 67		Pre-CRT SUV _{max} or MTV did not correlate with outcome
³¹³	20	pCT	50.4 Gy in 28 # Capecitabine or UFT	Dworak TRG 3-4	Pre-CRT: BF < 59.25 mL/100 g/min MTT > 9.52 s	80, 73 59, 92 100, 87 71, 100	0.880 0.920	11/20 has imaging both pre- and post CRT. 20/20 had pre-CRT pCT – there was no difference in the changes pre- and post CRT to differentiate responders from non- responders
³¹⁴	30	dw-MRI	45 Gy in 25 # 5-FU	pCR				No significant difference in ADC between responders and non-responders
³¹⁵	50	FDG-PET	50.4 Gy in 28 # 5-FU	Fibrosis:viable tumour ≥ 0.51	%ΔSUV _{max} ≥ 53	75, 73 83, 62	0.762	
³¹⁶	35	dw-MRI	50.4 Gy in 28 # 5-FU	T downstaging	Post-ADC > 1.18×10^{-3}	91, 50 78, 75		

					Absolute $\Delta\text{ADC} > 0.12 \times 10^{-3}$	96, 50 79, 86		
³¹⁷	99	FDG-PET	54 Gy in 30 # 5-FU	Clinical or pathological CR	Visual assessment	93, 53 87, 73		12 weeks post-CRT
⁷²	70	FDG-PET	45-55 Gy in 25 # 5-FU or capecitabine	pCR	$\%\Delta\text{SUV}_{\text{max}} \geq 63$	60, 84 92, 42	0.757	
					Post-CRT $\text{SUV}_{\text{max}} < 4$	58, 78 88, 39	0.723	
³¹⁸	38	FDG-PET	Induction 5-FU + oxaliplatin 45-50.4 Gy in 25-28 # +/- 10-12.5 Gy IORT Tegafur	Dworak TRG 3-4	Pre- $\text{SUV}_{\text{max}} < 6$	79, 84 83, 80		Validation of pre-defined imaging parameter cut-offs
					Post- $\text{SUV}_{\text{max}} < 2$	68, 79 77, 71		
					Absolute $\Delta\text{SUV}_{\text{max}} \geq 4$	79, 63 68, 75		
					$\%\Delta\text{SUV}_{\text{max}} \geq 65$	74, 79 78, 75		
³¹⁹	28	dw-MRI	50.4 Gy in 28 # 5-FU and cisplatin	pCR	% increase in ADC > 29.5	83, 90 91, 82	0.913	
					Post-CRT ADC		0.875	
³²⁰	121	FDG-PET	45-54 Gy, fractionation NR 5-FU or 5-FU + cetuximab or 5-FU + irinotecan	pCR	Pre-CRT SUV_{max} , SUV_{mean} , TLG, MV		0.520- 0.620	
					$\%\Delta\text{SUV}_{\text{max}}$		0.730	
					$\%\Delta\text{SUV}_{\text{mean}}$		0.690	
					$\%\Delta\text{TLG}$		0.520	
					Visual assessment	54, 66 30, 84		

321	59	dw-MRI	50 Gy in 25 # Capecitabine	pCR	Pre-ADC < 0.97×10^{-3}	56, 86 42, 91	0.770	
				Mandard TRG 1-2	% increase in ADC > 41	78, 92 64, 96	0.950	
					Pre-ADC < 0.97×10^{-3}	62, 91 67, 89	0.850	
					% increase in ADC > 41	77, 98 91, 94	0.960	
59	151	FDG-PET	50.4 Gy, fractionation NR 5-FU or capecitabine or tegafur-uracil	pCR	Post-CRT SUV _{max} < 3.55	74, 64 23, 94	0.689	% was significantly larger in those who were downstaged (69% vs 69% p = 0.007) Independent predictive utility not assessed
				T downstaging to ypT0-1	Post-CRT SUV _{max} < 3.55 and post-CRT CEA < 1.65 ng/mL	42, 92 42, 92	0.773	
					Post-CRT SUV _{max} < 3.7	60, 65 45, 78	0.623	
					Post-CRT SUV _{max} < 3.7 and post-CRT CEA < 5.3 ng/mL	52, 84 59, 78	0.715	
322	31	dw-MRI	50.4 Gy in 28 # Capecitabine or 5-FU +/- oxaliplatin	Mandard TRG 1-2	Post-CRT ADC > 1.294×10^{-3}	86, 67 86, 67	0.820	Measurements taken by 2 independent readers. As intraclass correlation coefficient was 1.0, the results from one reader alone are presented Pre-CRT ADC was not different in responders, cf non-responders
					Absolute ADC increase > 0.50×10^{-3}	91, 67 87, 75	0.830	
					% increase in ADC > 41	64, 67 82, 43	0.680	

³²³	41	FDG-PET	50-58 Gy, fractionation NR Capecitabine	Mandard TRG 3-5	Visual assessment	89, 93 96, 81		% reduction in SUV _{max} in responders 79.9 vs 60.3 in non-responders (p < 0.037)
⁸³	91	dce-MRI	50.4 Gy in 28 # 5-FU	T and N downstaging				A measure of intravascular-interstitial contrast exchange decreased post-CRT. The relative volume of the interstitial space increased post CRT.
⁷¹	61	FDG-PET	50.4 Gy in 28 # (25 Gy in 5 # in 7 patients) 5-FU or tegafur or capecitabine	Dworak TRG 3-4	Post-SUV _{max} < 3.7 %ΔSUV _{max} ≥ 67.9 %ΔSUV _{max} ≥ 5.7 from 60-90 min on post-CRT FDG-PET	67, 61 34, 85 60, 72 41, 85 87, 87 68, 95	0.703 0.696 0.906	Comparing the SUV _{max} at 60 and 90 min post-injections led to a significantly improved ROC curve AUC (p = 0.02)
⁸⁵	16	FDG-PET and pCT	45 Gy in 25 # 5-FU	Dworak TRG 3-4	%ΔFMP > 75	100, 91 67, 100	0.955	FMP = FDG SUV _{max} x pCT BF
³²⁴	181	FDG-PET	45 Gy in 25 # 5-FU	pCR	%ΔSUV _{max} ≥ 63.6	73, 65 26, 94	0.723	

Table V: Prognostic utility of functional imaging in LARC

Ref	n	Imaging modality	Total tumour radiation dose and chemotherapy agents	Median follow-up	Survival end-point	Imaging parameter	Sens,Spec PPV, NPV (%)	AUC	Comments
Imaging pre-CRT only									
²⁸⁴	81	FDG-PET	45 Gy in 25 # 5-FU	26.7 months	DFS	TLG > median			HR 20.0 (95% CI 1.7-232.6 p = 0.017)
⁴⁷	49	dw-MRI	45-50.4 Gy over mean of 6 weeks 5-FU +/- oxaliplatin or capecitabine	16.4 months	Recurrence	ADC > 0.833 x 10 ⁻³ Reader 1 Reader 2	86, 77 67, 91 80, 69 58, 87	0.856 0.791	ADC was lower in those who showed local recurrence (p < 0.001)
²⁸⁵	38	FDG-PET	Induction 5-FU + oxaliplatin 45-50.4 Gy in 25-28 # Tegafur 10-12.5 Gy intraoperatively	69.3 months	5 yr DFS OS	SUV _{max} ≥ 8.4 and overexpression of VEGFR- 2 SUV _{max} ≥ 8.4 and overexpression of VEGFR- 2			89.4% vs 52.9% (p = 0.01) HR 4.73 HR 4.28
³²⁵	17	Cu-ATSM- PET	45 Gy in 25 # 5-FU	4.1 years	3 yr PFS 3 yr OS	T:M > 2.6 (median)			50% vs 100% (p < 0.05) 63% vs 100% (p < 0.05)
Imaging pre- and during-CRT									
²⁹¹	27	FDG-PET	50.4 Gy in 28 # 5-FU +/- cetuximab	40 months	DFS	VRS > 50%			100% DFS if >50% response in FDG-PET 20% of those with <50% response recurred
⁶⁹	42	FDG-PET	45 Gy in 25 # Induction oxaliplatin followed by 5-FU based CRT	91 months	5 yr RFS	%ΔSUV _{mean} ≥ 52 %ΔSUV _{max} ≥ 42			86% vs 55% (p = 0.02), HR 0.22 (95% CI 0.06-0.83)

									HR 0.41 (95% CI 0.13-1.30) FDG-PET pre- and after 12 days of CRT
Imaging pre- and post-CRT									
³²⁶	15	FDG-PET	50.4 Gy, fractionation NR 5-FU	42 months	No evidence of disease	% Δ SUV _{max} $\geq$ 62.5 Δ TLG $\geq$ 69.5%	64, 100 -, - 82, 75 -, -		Those with metastatic disease detected on repeat imaging excluded from analysis Mean Δ SUV _{max} in the survivor group 68.5% vs 36.8% in those with recurrent disease (p = 0.004)
³²⁷	30	FDG-PET	50.4 Gy in > 10 # 5-FU or capecitabine (Also carboplatin in 1 and oxaliplatin in 2 patients)	3.1 years	3 yr OS 3 yr PFS	Qualitative visual assessment			100% mCR, 79% mPR, 0% mSD/mPD (p < 0.0001) 100% mCR, 47% mPR, 0% mSD/mPD (p < 0.0001)
³⁰³	53	FDG-PET	50.4-54 Gy in 28-30 # or IMRT in 2.2 Gy #s, total dose NR Capecitabine or 5-FU or 5-FU and MMC	31 months	DFS	Baseline SUV _{max} , % Δ SUV _{max}			Neither PET parameter was associated with DFS
³⁰⁵	20	FDG-PET	50.4-54 Gy in 28-30 # 5-FU	5 years		Qualitative visual assessment			3/3 patients with no PET response died of disease 7/7 mCR free of disease 7/13 (54%) incomplete metabolic response developed distant disease
³¹¹	80	FDG-PET	50.4 Gy in 28 # 5-FU +/- oxaliplatin, 5-FU +/- panitumumab	44 months	DFS	Post SUV _{max} < 5			Pre- SUV _{max} and % Δ SUV _{max} did not correlate with outcome

									HR of any recurrence 11.3 (95% CI 1.8-71.5) if Post SUV _{max} > 5
328	75	FDG-PET	50.4 Gy in 28 # 5-FU	3.1 years	OS DFS	Visual assessment No response No response			HR 10.2 HR 5.8
329	127	FDG-PET	45-54 Gy, fractionation NR 5-FU, 5-FU + cetuximab, 5- FU + irinotecan		DFS DSS	Absolute and relative changes in SUV _{max} , SUV _{mean} , TLG, visual response			No parameter was associated with time to recurrence of disease specific survival
72	70	FDG-PET	45-55 Gy in 25 # 5-FU or capecitabine	40 months	OS	Post SUV _{max} < 4 %ΔSUV _{max} ≥ 63			OR 7.4 (95% CI 1.7-33) OR 14.9 (95% CI 2.6-87)
318	38	FDG-PET	Induction 5-FU + oxaliplatin 45-50.4 Gy in 25-28 # +/- 10-12.5 Gy IORT Tegafur	69.3 months	5 yr DFS 5 yr OS	%ΔSUV _{max} ≥ 65 Absolute ΔSUV _{max} < 4 %ΔSUV _{max} < 65 Absolute ΔSUV _{max} < 4			94.1% vs 51.6% (p = 0.003) 94.4% vs 48.8% (p = 0.002) 94.1% vs 65.0% (p = 0.02) 94.4% vs 63.2% (p = 0.01)
Post-CRT imaging only									
298	21	FDG-PET	45-50.4 Gy in 25-28 # 5-FU or tegafur		3 yr OS	SUV _{max} ≥ 6.0			60% vs 93% (p = 0.04) Post-CRT imaging non- discriminatory
330	88	FDG-PET		38 months	5 yr OS	Visual assessment 'positive' or 'negative'			81% in those with 'negative' FDG-PET vs 62% if residual FDG uptake (p = 0.024)

Appendix B

Texture matrices and features

	Ref NO.	Name	Governing equations derived from references:
First Order Statistic	m1	mean (normalised)	245,331-333
	v1	variance	
	sd1	standard deviation	
	s1	entropy	
	cov1	coefficient of variation (sd/m) (normalised)	
	max1	maximum value of image (normalised)	
	min1	minimum value of image (normalised)	
	vol1	volume (use the number of pixels to represent)	
	diax	diameter of the ROI/lesion, x direction	
	diay	diameter of the ROI/lesion, y direction	
	skew	the skewness of the image	
	kurt	the kurtosis of the image	
	m2	mean (original)	
	cov2	coefficient of variation (sd/m) (original)	
	max2	maximum value of image (original)	
	min2	minimum value of image (original)	
Grey-level Co-occurrence Matrix	HM	Homogeneity	247,257,331,334
	EG	Energy	
	CV	Covariance	
	CR	Correlation	
	CT	Contrast	
	ET	Entropy	
	DS	Dissimilarity	
Grey-level Run Length Matrix OR Grey-level Size Zone Matrix	SRE	Short Run Emphasis	248,249,331,335
	LRE	Long Run Emphasis	
	GLN	Grey-Level Non-uniformity	
	RLN	Run Length Non-uniformity	
	RP	Run Percentage	
	LGRE	Low Grey-level Run Emphasis	
	HGRE	High Grey-level Run Emphasis	
	SRLGE	Short Run Low Grey-level Emphasis	
	SRHGE	Short Run High Grey-level Emphasis	
	LRLGE	Long Run Low Grey-level Emphasis	
	LRHGE	Long Run High Grey-level Emphasis	
Neighbouring Grey-level Dependence Matrix	SNE	Small Number Emphasis	250,336
	LNE	Large Number Emphasis	
	NNU	Number Non-Uniformity	
	SCM	Second Moment	
	ENT	Entropy	
Neighbourhood Grey-tone Difference Matrix	COA	Coarseness	251
	CON	Contrast	
	BSY	Busyness	

	COM	Complexity	
	TST	Texture Strength	

Mathematical expression

	Ref NO.	
First Order Statistic	m1	$g^T p$
	v1	$(diag(g - m1)(g - m1))^T p$
	sd1	$\sqrt{v1}$
	s1	$-p^T(\log_2 p)$
	cov1	$sd1/m1$
	max1	$max(im)$
	min1	$min(im)$
	vol1	$dim(im)$
	diax	* [x, ~]=dia(image)
	diay	* [~, y]=dia(image)
	skew	$\left((diag(g - m1))^2 (g - m1) \right)^T p / sd1^3$
	kurt	$\left((diag(g - m1))^3 (g - m1) \right)^T p / sd1^4$
	m2	See m1
	cov2	See cov1
	max2	See max1
	min2	See min1
g: grey-level vector		
p: probability vector of grey level, i.e. $\frac{n}{n^T n}$		
n: number of pixels in matrix		
im: image matrix in pixel		
Grey-level Co-occurrence Matrix	HM	$\sum_{i,j} \frac{p(i,j)}{(1+ i-j)} \text{ or } \sum_{i,j} \frac{p(i,j)}{(1-(i-j)^2)} \text{ (Ref. 8)}$
	EG	$\sum_{i,j} p(i,j)^2$
	CV	$\sum_{r,c} (r - u_r)(c - u_c)p(r) \text{ where, } u_r = \sum_r rp(r), u_c = \sum_c cp(c)$
	CR	$\frac{cv}{(\sigma_r \sigma_c)} \text{ where, } \sigma_r = \sqrt{\sum_r ((r - u_r)^2 p(r))}, \sigma_c = \sqrt{\sum_c ((c - u_c)^2 p(c))}$
	CT	$\sum_{i,j} ((i - j)^2 p(i,j))$
	ET	$-\sum_{i,j} p(i,j) \log_2 p(i,j)$
	DS	$\sum_{i,j} i - j p(i,j)$
co: Co-occurrence matrix		
i, j: Row and column indices of the matrix		
r, c: Row and column subscript sets of matrix		
p: Normalised co-occurrence matrix $p(i,j)$ or vector $p(r)$, i.e., $\frac{co}{\sum_{i,j} co(i,j)}$		
Grey-level Run Length Matrix OR Grey-level Size Zone Matrix	SRE	$\frac{1}{n} \sum_{i,j} \frac{G(i,j)}{j^2}$
	LRE	$\frac{1}{n} \sum_{i,j} G(i,j) * j^2$
	GLN	$\frac{1}{n} \sum_i (\sum_j G(i,j))^2$
	RLN	$\frac{1}{n} \sum_j (\sum_i G(i,j))^2$
	RP	$\frac{n}{i*j}$
	LGRE	$\frac{1}{n} \sum_{i,j} \frac{G(i,j)}{i^2}$
	HGRE	$\frac{1}{n} \sum_{i,j} (G(i,j) * i^2)$

	SRLGE	$\frac{1}{n} \sum_{i,j} \frac{G(i,j)}{(i*j)^2}$
	SRHGE	$\frac{1}{n} \sum_{i,j} \frac{G(i,j)*i^2}{j^2}$
	LRLGE	$\frac{1}{n} \sum_{i,j} \frac{G(i,j)*j^2}{i^2}$
	LRHGE	$\frac{1}{n} \sum_{i,j} (G(i,j) * i^2 * j^2)$
G: Run Length (or Size Zone) matrix		
i, j: Row and column indices of the matrix		
n: Total number of Runs in the matrix (i.e. $\sum_{i,j} G$)		
Neighbouring Grey-level Dependence Matrix	SNE	$\frac{\sum_{i,j} \frac{N(i,j)}{j^2}}{\sum_{i,j} N(i,j)}$
	LNE	$\frac{\sum_{i,j} N(i,j)*j^2}{\sum_{i,j} N(i,j)}$
	NNU	$\frac{\sum_i (\sum_j N(i,j))^2}{\sum_{i,j} N(i,j)}$
	SCM	$\frac{\sum_{i,j} N(i,j)^2}{\sum_{i,j} N(i,j)}$
	ENT	$-\sum_{i,j} \frac{N(i,j)*\log_2 N(i,j)}{s}$
N: Grey-level Dependence Matrix		
i, j: Row and column indices of the matrix		
s: Sum of the matrix (i.e., $\sum_{i,j} N$)		
Neighbourhood Grey-tone Difference Matrix	COA	$\frac{1}{e+p^T s}$
	CON	$\frac{\sum_{i,j} (p(i)p(j)(i-j)^2)}{n(n-1)} * \frac{\sum_i s(i)}{w}$
	BSY	$\frac{2*\sum_i (p(i)s(j))}{\sum_{i,j} ip(i)-jp(j) }$
	COM	$\sum_{i,j} \frac{ i-j }{w(p(i)+p(j))} * (p(i)s(i) + p(j)s(j))$
	TST	$\frac{\sum_{i,j} ((p(i)+p(j))(i-j)^2)}{e+\sum_i s(i)}$
i, j: grey-tone level indices		
n: total number of grey-tone levels		
w: number of pixels processed in ROI matrix		
p: probability vector of grey-tone level occurrences in the normalised ROI matrix		
e: 10^{-6} , to prevent division by zero		
s: vector of total grey-tone difference from neighbours for each grey-tone level		

Appendix C

Blood samples

Collect blood in EDTA (purple-topped) vacutainer and process within 30 min. Maintain samples below 4°C before processing (on wet ice). Centrifuge at 2000xg for 10 min at 4°C. Set braking speed to the lowest setting. Note the time at which centrifugation commenced in the sample log. Using a Pasteur pipette, carefully transfer the plasma layer (~5 mL) to a fresh tube without disturbing the buffy coat layer. (The buffy coat layer should be aspirated and retained and stored at $\leq -60^{\circ}\text{C}$ (removing some of the red blood cells with the buffy coat is acceptable.) Discard the red blood cells. Centrifuge plasma at 2000xg for 10 min at 4°C. Label two ~2 mL cryovials 'Plasma for ELISA' and one 'Plasma for ctDNA'. Carefully transfer the plasma layer to a fresh tube without disturbing the pellet and aliquot ~ equal volumes between three 2 mL cryovials and store at $\leq -60^{\circ}\text{C}$.

Rectal tumour biopsy material

RNAlater® sample

Refrigerate the biopsy sample as soon as possible at 4°C and leave for 24–72 h. Up to 72 h later remove the sample from the RNAlater® using RNAase free forceps and place sample into a correctly labelled 1.5 mL cryovial. Freeze at $\leq -60^{\circ}\text{C}$ in the labelled cryovial.

RNA extraction

Cryovials were allowed to thaw at room temperature. RNAlater® solution was removed and replaced with 500 μL Trizol. Samples were then placed on ice, homogenized for 30 s on Level 4 setting using the Tissue Tearer Homogenizer (BioSpec Products Inc., Bartlesville, OK, USA) before incubating on ice again. Samples were incubated at room temperature for 5 min, then 50 μL BCP (1-bromochloro-3-chloropropane – chloroform specific for RNA extraction) was added to the tube, agitated vigorously by hand for 15 s before incubating at room temperature for 3 min. Samples were then centrifuged at 12,000xg for 15 min at 4°C. The top aqueous phase (~200 μL) transferred to a separate tube containing 1 μL glycogen (Invitrogen, Life Technologies). RNA was precipitated by mixing with 200 μL (equivolume to aqueous phase) isopropanol (molecular biology grade; filtered). Samples were vortexed and incubated samples at room temperature for 10 min. The supernatant was discarded and the RNA pellet washed with 0.75 ml 75% ethanol (molecular biology grade; filtered) and vortexed again. Samples were centrifuged at 7500xg for 5 min at 4°C. The supernatant was discarded and the RNA pellet was air dried for 10 min in a tissue culture hood. The RNA pellet was redissolved in RNase-free water (20 μL) and incubated for 10 min at 60°C before refreezing at -80°C .

Formalin-fixed sample

Leave at room temperature for 24–72 h without disturbing. Then remove sample from formalin and place it in 70% ethanol. Samples were then embedded in paraffin and sectioned prior to staining with H&E as per standard clinical protocols.

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References

- 1 Northover, J. M. A. *et al.* Epidermoid anal cancer: Results from the UKCCCR randomised trial of radiotherapy alone versus radiotherapy, 5-fluorouracil, and mitomycin. *Lancet* **348**, 1049-1054 (1996).
- 2 Pignon, J. P., Maître, A. I., Maillard, E. & Bourhis, J. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): An update on 93 randomised trials and 17,346 patients. *Radiother Oncol.* **92**, 4-14 (2009).
- 3 Vale, C. *et al.* Reducing uncertainties about the effects of chemoradiotherapy for cervical cancer: A systematic review and meta-analysis of individual patient data from 18 randomized trials. *J Clin Oncol* **26**, 5802-5812 (2008).
- 4 Sauer, R. *et al.* Preoperative versus postoperative chemoradiotherapy for rectal cancer. *New England Journal of Medicine* **351**, 1731-1740+1810 (2004).
- 5 CRUK. *Pancreatic cancer survival statistics*, <<http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer>> (Accessed 14th January 2015)
- 6 Bosset, J. F. *et al.* Preoperative chemoradiotherapy versus preoperative radiotherapy in rectal cancer patients: Assessment of acute toxicity and treatment compliance: Report of the 22921 randomised trial conducted by the EORTC Radiotherapy Group. *Eur J Cancer* **40**, 219-224 (2004).
- 7 Grégoire, V., Jeraj, R., Lee, J. A. & O'Sullivan, B. Radiotherapy for head and neck tumours in 2012 and beyond: Conformal, tailored, and adaptive? *Lancet Oncol* **13**, 1 (2012).
- 8 Gray, L. H., Conger, A. D., Ebert, M., Hornsey, S. & Scott, O. C. A. The Concentration of Oxygen Dissolved in Tissues at the Time of Irradiation as a Factor in Radiotherapy. *Br J Radiol* **26**, 638-648 (1953).
- 9 Koukourakis, M. I., Giatromanolaki, A., Sivridis, E. & Fezoulidis, I. Cancer vascularization: Implications in radiotherapy? *Int J Radiat Oncol Biol Phys* **48**, 545-553 (2000).
- 10 Höckel, M. *et al.* Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* **56**, 4509-4515 (1996).
- 11 Nordsmark, M. *et al.* Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. *Radiother Oncol* **77**, 18-24 (2005).
- 12 Milosevic, M. *et al.* Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clin Cancer Res* **18**, 2108-2114 (2012).
- 13 Lu, X. & Kang, Y. Hypoxia and hypoxia-inducible factors: Master regulators of metastasis. *Clin Cancer Res* **16**, 5928-5935 (2010).
- 14 Wilson, W. R. & Hay, M. P. Targeting hypoxia in cancer therapy. *Nat Rev Cancer* **11**, 393-410 (2011).
- 15 Harris, A. L. Hypoxia - A key regulatory factor in tumour growth. *Nat Rev Cancer* **2**, 38-47 (2002).
- 16 Semenza, G. L. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**, 721-732 (2003).

- 17 CRUK. Cancer statistics. <http://www.cancerresearchuk.org/cancer-info/cancerstats/types/pancreas/>. (Accessed 15th January 2015)
- 18 Yeo, T. P. *et al.* Pancreatic cancer. *Curr Probl Cancer* **26**, 176-275 (2002).
- 19 Mukherjee, S. *et al.* Gemcitabine-based or capecitabine-based chemoradiotherapy for locally advanced pancreatic cancer (SCALOP): A multicentre, randomised, phase 2 trial. *Lancet Oncol* **14**, 317-326 (2013).
- 20 Hammel, P. *et al.* Randomized multicenter phase III study in patients with locally advanced adenocarcinoma of the pancreas: gemcitabine with or without chemoradiotherapy and with or without erlotinib - LAP07 study. *ASCO Meeting Abstracts* **29**, e14619 (2013).
- 21 Crane, C. H. *et al.* Phase II trial of cetuximab, gemcitabine, and oxaliplatin followed by chemoradiation with cetuximab for locally advanced (T4) pancreatic adenocarcinoma: Correlation of Smad4(Dpc4) immunostaining with pattern of disease progression. *J Clin Oncol* **29**, 3037-3043 (2011).
- 22 Krishnan, S. *et al.* Induction chemotherapy selects patients with locally advanced, unresectable pancreatic cancer for optimal benefit from consolidative chemoradiation therapy. *Cancer* **110**, 47-55 (2007).
- 23 Lacobuzio-Donahue, C. A. *et al.* DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. *J Clin Oncol* **27**, 1806-1813 (2009).
- 24 Loehrer Sr, P. J. *et al.* Gemcitabine alone versus gemcitabine plus radiotherapy in patients with locally advanced pancreatic cancer: An Eastern Cooperative Oncology Group trial. *J Clin Oncol* **29**, 4105-4112 (2011).
- 25 White, R. R. *et al.* Significance of histological response to preoperative chemoradiotherapy for pancreatic cancer. *Ann Surg Oncol* **12**, 214-221 (2005).
- 26 Ritts, R. E. & Pitt, H. A. CA 19-9 in pancreatic cancer. *Surg Oncol Clin N Am* **7**, 93-101 (1998).
- 27 Vainshtein, J. M. *et al.* Prognostic significance of carbohydrate antigen 19-9 in unresectable locally advanced pancreatic cancer treated with dose-escalated intensity modulated radiation therapy and concurrent full-dose gemcitabine: Analysis of a prospective phase 1/2 dose escalation study. *Int J Radiat Oncol Biol Phys* **86**, 96-101 (2013).
- 28 Yang, G. Y. *et al.* Change in CA 19-9 levels after chemoradiotherapy predicts survival in patients with locally advanced unresectable pancreatic cancer. *J Gastrointest Oncol* **4**, 361-369 (2013).
- 29 CRUK. UK Cancer Incidence 2011 and mortality 2011 summary – Counts. <http://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence> (Accessed 15th January 2015).
- 30 Brown, G. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: Prospective observational study. *British Medical Journal* **333**, 779-782 (2006).
- 31 Bosset, J. F. *et al.* Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* **355**, 1114-1123 (2006).
- 32 Gérard, J. P. *et al.* Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: Results of FFCD 9203. *J Clin Oncol* **24**, 4620-4625 (2006).

- 33 Maas, M. *et al.* Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: A pooled analysis of individual patient data. *Lancet Oncol* **11**, 835-844 (2010).
- 34 Habr-Gama, A. *et al.* Long-term results of preoperative chemoradiation for distal rectal cancer correlation between final stage and survival. *J Gastrointest Surg* **9**, 90-101 (2005).
- 35 O'Neill, B. D., Brown, G., Heald, R., Cunningham, D. & Tait, D. M. Non-operative treatment after neoadjuvant chemoradiotherapy for rectal cancer. *Lancet Oncol.* **8**, 625-633 (2007).
- 36 Wolthuis, A. M. *et al.* Impact of interval between neoadjuvant chemoradiotherapy and TME for locally advanced rectal cancer on pathologic response and oncologic outcome. *Ann Surg Oncol* **19**, 2833-2841 (2012).
- 37 Bennis, M. *et al.* Morbidity risk factors after low anterior resection with total mesorectal excision and coloanal anastomosis: A retrospective series of 483 patients. *Ann Surg* **255**, 504-510 (2012).
- 38 Kelly, P. *et al.* Duodenal toxicity after fractionated chemoradiation for unresectable pancreatic cancer. *Int J Radiat Oncol Biol Phys* **85**, e143-e149 (2013).
- 39 Kim, Y. H. *et al.* Usefulness of magnetic resonance volumetric evaluation in predicting response to preoperative concurrent chemoradiotherapy in patients with resectable rectal cancer. *Int J Radiat Oncol Biol Phys* **62**, 761-768 (2005).
- 40 Kim, N. K. *et al.* A comparative study of volumetric analysis, histopathologic downstaging, and tumor regression grade in evaluating tumor response in locally advanced rectal cancer following preoperative chemoradiation. *Int J Radiat Oncol Biol Phys* **67**, 204-210 (2007).
- 41 Patel, U. B. *et al.* Comparison of magnetic resonance imaging and histopathological response to chemoradiotherapy in locally advanced rectal cancer. *Ann Surg Oncol* **19**, 2842-2852 (2012).
- 42 Habr-Gama, A. *et al.* Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: Long-term results. *Ann Surg* **240**, 711-718 (2004).
- 43 Smith, R. K., Fry, R. D., Mahmoud, N. N. & Paulson, E. C. Surveillance after neoadjuvant therapy in advanced rectal cancer with complete clinical response can have comparable outcomes to total mesorectal excision. *Int J Colorectal Dis* **30**, 769-774 (2015).
- 44 Chang, J. S. *et al.* Clinical usefulness of 18F-fluorodeoxyglucose-positron emission tomography in patients with locally advanced pancreatic cancer planned to undergo concurrent chemoradiation therapy. *Int J Radiat Oncol Biol Phys* **90**, 126-133 (2014).
- 45 Davey, K. *et al.* The impact of 18-fluorodeoxyglucose positron emission tomography-computed tomography on the staging and management of primary rectal cancer. *Dis Colon Rectum* **51**, 997-1003 (2008).
- 46 Bassi, M. C. *et al.* FDG-PET/CT imaging for staging and target volume delineation in preoperative conformal radiotherapy of rectal cancer. *Int J Radiat Oncol Biol Phys* **70**, 1423-1426 (2008).
- 47 Elmi, A. *et al.* Apparent diffusion coefficient as a non-invasive predictor of treatment response and recurrence in locally advanced rectal cancer. *Clin Radiol* **68**, e524-531 (2013).

- 48 Lambrecht, M. *et al.* The use of FDG-PET/CT and diffusion-weighted magnetic resonance imaging for response prediction before, during and after preoperative chemoradiotherapy for rectal cancer. *Acta Oncol* **49**, 956-963 (2010).
- 49 Wieder, H. A. *et al.* PET imaging with [18F]3'-deoxy-3'-fluorothymidine for prediction of response to neoadjuvant treatment in patients with rectal cancer. *Eur J Nucl Med Mol Imaging* **34**, 878-883 (2007).
- 50 Dehdashti, F. *et al.* Positron emission tomography with [(18)F]-3'-deoxy-3'-fluorothymidine (FLT) as a predictor of outcome in patients with locally advanced resectable rectal cancer: a pilot study. *Mol. Imaging Biol.* **15**, 106-113 (2013).
- 51 Wieder, H. *et al.* PET imaging with [11C]methyl- L-methionine for therapy monitoring in patients with rectal cancer. *Eur J Nucl Med Mol Imaging* **29**, 789-796 (2002).
- 52 Mandard, A. M. *et al.* Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma: Clinicopathologic correlations. *Cancer* **73**, 2680-2686 (1994).
- 53 Dworak, O., Keilholz, L. & Hoffmann, A. Pathological features of rectal cancer after preoperative radiochemotherapy. *Int J Colorectal Dis* **12**, 19-23 (1997).
- 54 Krenkli, M. *et al.* Target volume delineation for preoperative radiotherapy of rectal cancer: inter-observer variability and potential impact of FDG-PET/CT imaging. *Technol. Cancer Res Treat* **9**, 393-398 (2010).
- 55 Kittaka, H. *et al.* Role of (18)F-fluorodeoxyglucose positron emission tomography/computed tomography in predicting the pathologic response to preoperative chemoradiation therapy in patients with resectable T3 pancreatic cancer. *World J Surg* **37**, 169-178 (2013).
- 56 Barbaro, B. *et al.* Diffusion-weighted magnetic resonance imaging in monitoring rectal cancer response to neoadjuvant chemoradiotherapy. *Int J Radiat Oncol Biol Phys* **83**, 594-599 (2012).
- 57 Prasad, D. S., Scott, N., Hyland, R., Guthrie, J. A. & Tolan, D. J. Diffusion-weighted MR imaging for early detection of tumor histopathologic downstaging in rectal carcinoma after chemotherapy and radiation therapy. *Radiology* **256**, 671-672; author reply 672 (2010).
- 58 Vecchio, F. M. *et al.* The relationship of pathologic tumor regression grade (TRG) and outcomes after preoperative therapy in rectal cancer. *Int J Radiat Oncol Biol Phys* **62**, 752-760 (2005).
- 59 Kim, J. W. *et al.* Predictive value of 18FDG PET-CT for tumour response in patients with locally advanced rectal cancer treated by preoperative chemoradiotherapy. *Int J Colorectal Dis*, **28**, 1-8 (2013).
- 60 Marcus, C. *et al.* Head and Neck PET/CT: Therapy Response Interpretation Criteria (Hopkins Criteria)—Interreader Reliability, Accuracy, and Survival Outcomes. *J Nucl Med*, **55**, 1411-6 (2014).
- 61 Kim, S. H. *et al.* Locally advanced rectal cancer: added value of diffusion-weighted MR imaging in the evaluation of tumor response to neoadjuvant chemo- and radiation therapy. *Radiology* **253**, 116-125 (2009).
- 62 DeVries, A. F. *et al.* Tumor microcirculation and diffusion predict therapy outcome for primary rectal carcinoma. *Int J Radiat Oncol Biol Phys* **56**, 958-965 (2003).

- 63 Lambregts, D. M. *et al.* Tumour ADC measurements in rectal cancer: effect of ROI methods on ADC values and interobserver variability. *Eur Radiol* **21**, 2567-2574 (2011).
- 64 Song, I. *et al.* Value of diffusion-weighted imaging in the detection of viable tumour after neoadjuvant chemoradiation therapy in patients with locally advanced rectal cancer: comparison with T2 weighted and PET/CT imaging. *Br J Radiol* **85**, 577-586 (2012).
- 65 Wahl, R. L., Jacene, H., Kasamon, Y. & Lodge, M. A. From RECIST to PERCIST: Evolving considerations for PET response criteria in solid tumors. *J Nucl Med* **50**, 122S-150S (2009).
- 66 Soret, M., Bacharach, S. L. & Buvat, I. Partial-volume effect in PET tumor imaging. *J Nucl Med* **48**, 932-945 (2007).
- 67 Hatt, M. *et al.* Early prediction of pathological response in locally advanced rectal cancer based on sequential 18F-FDG PET. *Acta Oncol* **52**, 619-626 (2013).
- 68 Cascini, G. L. *et al.* 18F-FDG PET is an early predictor of pathologic tumor response to preoperative radiochemotherapy in locally advanced rectal cancer. *J Nucl Med* **47**, 1241-1248 (2006).
- 69 Avallone, A. *et al.* Early FDG PET response assessment of preoperative radiochemotherapy in locally advanced rectal cancer: correlation with long-term outcome. *European J Nucl Med & Molecular Imaging* **39**, 1848-1857 (2012).
- 70 Tixier, F. *et al.* Intratumor heterogeneity characterized by textural features on baseline 18F-FDG PET images predicts response to concomitant radiochemotherapy in esophageal cancer. *J Nucl Med* **52**, 369-378 (2011).
- 71 Yoon, H. J. *et al.* New application of dual point 18F-FDG PET/CT in the evaluation of neoadjuvant chemoradiation response of locally advanced rectal cancer. *Clin Nucl Med* **38**, 7-12 (2013).
- 72 Shanmugan, S. *et al.* Predicting pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer using 18FDG-PET/CT. *Ann Surg Oncol* **19**, 2178-2185 (2012).
- 73 Vliegen, R. F. A. *et al.* Can an FDG-PET/CT predict tumor clearance of the mesorectal fascia after preoperative chemoradiation of locally advanced rectal cancer? *Strahlenther Onkol* **184**, 457-464 (2008).
- 74 Yoon, M. S. *et al.* The metabolic response using 18F-fluorodeoxyglucose-positron emission tomography/computed tomography and the change in the carcinoembryonic antigen level for predicting response to pre-operative chemoradiotherapy in patients with rectal cancer. *Radiother Oncol* **98**, 134-138 (2011).
- 75 Capirci, C. *et al.* The role of dual-time combined 18-fluorodeoxyglucose positron emission tomography and computed tomography in the staging and restaging workup of locally advanced rectal cancer, treated with preoperative chemoradiation therapy and radical surgery. *Int J Radiat Oncol Biol Phys* **74**, 1461-1469 (2009).
- 76 Hein, P. A. *et al.* Diffusion-weighted magnetic resonance imaging for monitoring diffusion changes in rectal carcinoma during combined, preoperative chemoradiation: Preliminary results of a prospective study. *Eur J Radiol* **45**, 214-222 (2003).

- 77 Lim, J. S. *et al.* Perfusion MRI for the prediction of treatment response after preoperative chemoradiotherapy in locally advanced rectal cancer. *Eur Radiol* **22**, 1693-1700 (2012).
- 78 Amthauer, H. *et al.* Response prediction by FDG-PET after neoadjuvant radiochemotherapy and combined regional hyperthermia of rectal cancer: correlation with endorectal ultrasound and histopathology. *Eur J Nucl Med Mol Imaging* **31**, 811-819 (2004).
- 79 Denecke, T. *et al.* Comparison of CT, MRI and FDG-PET in response prediction of patients with locally advanced rectal cancer after multimodal preoperative therapy: is there a benefit in using functional imaging? *Eur Radiol* **15**, 1658-1666 (2005).
- 80 Nakagawa, K. *et al.* Preoperative radiation response evaluated by 18-fluorodeoxyglucose positron emission tomography predicts survival in locally advanced rectal cancer. *Dis Colon Rectum* **51**, 1055-1060 (2008).
- 81 Lambregts, D. M. *et al.* Diffusion-weighted MRI for selection of complete responders after chemoradiation for locally advanced rectal cancer: a multicenter study. *Ann Surg Oncol* **18**, 2224-2231 (2011).
- 82 Capirci, C. *et al.* Restaging after neoadjuvant chemoradiotherapy for rectal adenocarcinoma: role of F18-FDG PET. *Biomed. Pharmacother.* **58**, 451-457 (2004).
- 83 Oberholzer, K. *et al.* Rectal cancer: assessment of response to neoadjuvant chemoradiation by dynamic contrast-enhanced MRI. *J Magn Reson Imaging* **38**, 119-126 (2013).
- 84 Janssen, M. H. M. *et al.* PET-based treatment response evaluation in rectal cancer: Prediction and validation. *Int J Radiat Oncol Biol Phys* **82**, 871-876 (2012).
- 85 Fischer, M. A. *et al.* Integrated 18F-FDG PET/perfusion CT for the monitoring of neoadjuvant chemoradiotherapy in rectal carcinoma: correlation with histopathology. *Eur J Nucl Med Mol Imaging* **41**, 1563-1573 (2014).
- 86 Tixier, F. *et al.* Correlation of intra-tumor 18F-FDG uptake heterogeneity indices with perfusion CT derived parameters in colorectal cancer. *PLoS One* **9**, e99567 (2014).
- 87 Sole, C. V. *et al.* Clinical significance of VEGFR-2 and 8F-FDG PET/CT SUVmax pretreatment score in predicting the long-term outcome of patients with locally advanced rectal cancer treated with neoadjuvant therapy. *Eur J Nucl Med Mol Imaging* **40**, 1635-1644 (2013).
- 88 Patel, D. A. *et al.* Impact of integrated PET/CT on variability of target volume delineation in rectal cancer. *Technol Cancer Res Treat* **6**, 31-36 (2007).
- 89 Geets, X., Lee, J. A., Bol, A., Lonneux, M. & Grégoire, V. A gradient-based method for segmenting FDG-PET images: Methodology and validation. *Eur J Nucl Med Mol Imaging* **34**, 1427-1438 (2007).
- 90 Roels, S. *et al.* Biological Image-Guided Radiotherapy in Rectal Cancer: Challenges and Pitfalls. *Int J Radiat Oncol Biol Phys* **75**, 782-790 (2009).
- 91 Havelund, B. M. *et al.* Tumour hypoxia imaging with 18F-fluoroazomycinarabinofuranoside PET/CT in patients with locally advanced rectal cancer. *Nucl Med Commun* **34**, 155-161 (2013).
- 92 Roels, S. *et al.* Biological image-guided radiotherapy in rectal cancer: Is there a role for FMISO or FLT, next to FDG? *Acta Oncol* **47**, 1237-1248 (2008).

- 93 Loi, S. *et al.* Oxaliplatin combined with infusional 5-fluorouracil and concomitant radiotherapy in inoperable and metastatic rectal cancer: A phase I trial. *Br J Cancer* **92**, 655-661 (2005).
- 94 Segard, T. *et al.* Detection of hypoxia with 18F-fluoromisonidazole (18F-FMISO) PET/CT in suspected or proven pancreatic cancer. *Clin Nucl Med* **38**, 1-6 (2013).
- 95 Choi, W. *et al.* Planning study for available dose of hypoxic tumor volume using fluorine-18-labeled fluoromisonidazole positron emission tomography for treatment of the head and neck cancer. *Radiother Oncol* **97**, 176-182 (2010).
- 96 Rasey, J. S., Koh, W. J., Grierson, J. R., Grunbaum, Z. & Krohn, K. A. Radiolabeled fluoromisonidazole as an imaging agent for tumor hypoxia. *Int J Radiat Oncol Biol Phys* **17**, 985-991 (1989).
- 97 Airley, R. *et al.* Glucose transporter Glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. *Clin Cancer Res* **7**, 928-934 (2001).
- 98 Schütze, C. *et al.* Effect of increase of radiation dose on local control relates to pre-treatment FDG uptake in FaDu tumours in nude mice. *Radiother Oncol* **83**, 311-315 (2007).
- 99 Aerts, H. J. W. L. *et al.* Identification of residual metabolic-active areas within NSCLC tumours using a pre-radiotherapy FDG-PET-CT scan: A prospective validation. *Lung Cancer* **75**, 73-76 (2012).
- 100 van den Bogaard, J. *et al.* Residual metabolic tumor activity after chemo-radiotherapy is mainly located in initially high FDG uptake areas in rectal cancer. *Radiother Oncol* **99**, 137-141 (2011).
- 101 Braendengen, M. *et al.* Delineation of gross tumor volume (GTV) for radiation treatment planning of locally advanced rectal cancer using information from MRI or FDG-PET/CT: a prospective study. *Int J Radiat Oncol Biol Phys* **81**, e439-445 (2011).
- 102 Paskeviciute, B. *et al.* Impact of (18)F-FDG-PET/CT on staging and irradiation of patients with locally advanced rectal cancer. *Strahlenther Onkol* **185**, 260-265 (2009).
- 103 Zips, D. *et al.* Exploratory prospective trial of hypoxia-specific PET imaging during radiochemotherapy in patients with locally advanced head-and-neck cancer. *Radiother Oncol* **105**, 21-28 (2012).
- 104 Thomlinson, R. H. & Gray, L. H. The Histological Structure of Some Human Lung Cancers and the Possible Implications for Radiotherapy. *Br J Cancer* **9**, 539-549 (1955).
- 105 Groebe, K. & Vaupel, P. Evaluation of oxygen diffusion distances in human breast cancer xenografts using tumor-specific in vivo data: Role of various mechanisms in the development of tumor hypoxia. *Int J Radiat Oncol Biol Phys* **15**, 691-697 (1988).
- 106 Olive, P. L., Vikse, C. & Trotter, M. J. Measurement of oxygen diffusion distance in tumor cubes using a fluorescent hypoxia probe. *Int J Radiat Oncol Biol Phys* **22**, 397-402 (1992).
- 107 Evans, S. M., Jenkins, W. T., Shapiro, M. & Koch, C. J. Evaluation of the concept of "hypoxic fraction" as a descriptor of tumor oxygenation status. *Adv. Exp. Med. Biol.* **411**, 215-225 (1997).

- 108 Barker, H. E., Paget, J. T. E., Khan, A. A. & Harrington, K. J. The tumour microenvironment after radiotherapy: Mechanisms of resistance and recurrence. *Nature Reviews Cancer* **15**, 409-425 (2015).
- 109 Brown, J. M. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *Br J Radiol* **52**, 650-656 (1979).
- 110 Dewhirst, M. W. *et al.* Microvascular studies on the origins of perfusion-limited hypoxia. *Br J Cancer* **74**, S247-S251 (1996).
- 111 Chaplin, D. J., Durand, R. E. & Olive, P. L. Acute hypoxia in tumors: Implications for modifiers of radiation effects. *Int J Radiat Oncol Biol Phys* **12**, 1279-1282 (1986).
- 112 Bayer, C., Shi, K., Astner, S. T., Maftai, C.-A. & Vaupel, P. Acute Versus Chronic Hypoxia: Why a Simplified Classification is Simply Not Enough. *Int J Radiat Oncol Biol Phys* **80**, 965-968 (2011).
- 113 Brizel, D. M., Sibley, G. S., Prosnitz, L. R., Scher, R. L. & Dewhirst, M. W. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* **38**, 285-289 (1997).
- 114 Brizel, D. M., Dodge, R. K., Clough, R. W. & Dewhirst, M. W. Oxygenation of head and neck cancer: Changes during radiotherapy and impact on treatment outcome. *Radiother Oncol* **53**, 113-117 (1999).
- 115 Fyles, A. W. *et al.* Oxygenation predicts radiation response and survival in patients with cervix cancer. *Radiother Oncol* **48**, 149-156 (1998).
- 116 Milosevic, M. *et al.* Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. *Clin Cancer Res* **18**, 2108-2114 (2012).
- 117 Hicks, R. J. *et al.* Utility of FMISO PET in advanced head and neck cancer treated with chemoradiation incorporating a hypoxia-targeting chemotherapy agent. *Eur J Nucl Med Mol Imaging* **32**, 1384-1391 (2005).
- 118 Rischin, D. *et al.* Tirapazamine, cisplatin, and radiation versus cisplatin and radiation for advanced squamous cell carcinoma of the head and neck (TROG 02.02, headstart): A phase III trial of the trans-tasman radiation oncology group. *J Clin Oncol* **28**, 2989-2995 (2010).
- 119 Toustrup, K. *et al.* Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. *Radiother Oncol* **102**, 122-129 (2012).
- 120 Rischin, D. *et al.* Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: A substudy of Trans-Tasman Radiation Oncology Group study 98.02. *J Clin Oncol* **24**, 2098-2104 (2006).
- 121 Overgaard, J., Eriksen, J. G., Nordsmark, M., Alsner, J. & Horsman, M. R. Plasma osteopontin, hypoxia, and response to the hypoxia sensitizer nimorazole in radiotherapy of head and neck cancer: Results from the DAHANCA 5 randomised double-blind placebo-controlled trial. *Lancet Oncol.* **6**, 757-764 (2005).
- 122 Overgaard, J. *et al.* A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. *Radiother Oncol* **46**, 135-146 (1998).

- 123 Hoogsteen, I. J., Marres, H. A. M., Bussink, J., Van Der Kogel, A. J. & Kaanders, J. H. A. M. Tumor microenvironment in head and neck squamous cell carcinomas: Predictive value and clinical relevance of hypoxic markers. A review. *Head Neck* **29**, 591-604, (2007).
- 124 Weiss, C. & Fleckenstein, W. Local tissue PO₂ measured with 'thick' needle probes. *Funktionsanal Biol Syst* **15**, 155-166 (1986).
- 125 Le, Q. T. *et al.* Comparison of the comet assay and the oxygen microelectrode for measuring tumor oxygenation in head-and-neck cancer patients. *Int J Radiat Oncol Biol Phys* **56**, 375-383 (2003).
- 126 Nordsmark, M. *et al.* Measurements of hypoxia using pimonidazole and polarographic oxygen-sensitive electrodes in human cervix carcinomas. *Radiother Oncol* **67**, 35-44 (2003).
- 127 Koukourakis, M. I. *et al.* Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. *J Clin Oncol* **24**, 727-735 (2006).
- 128 Toustrup, K. *et al.* Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. *Cancer Research* **71**, 5923-5931 (2011).
- 129 Chia, S. K. *et al.* Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J Clin Oncol* **19**, 3660-3668 (2001).
- 130 Wang, G. L., Jiang, B. H., Rue, E. A. & Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5510-5514 (1995).
- 131 Maxwell, P. H. *et al.* The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**, 271-275, (1999).
- 132 Jewell, U. R. *et al.* Induction of HIF-1alpha in response to hypoxia is instantaneous. *FASEB J.* **15**, 1312-1314 (2001).
- 133 Vordermark, D. *et al.* Glucose requirement for hypoxic accumulation of hypoxia-inducible factor-1alpha (HIF-1alpha). *Cancer Lett.* **230**, 122-133 (2005).
- 134 Janssen, H. L. *et al.* HIF-1A, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors. *Int J Radiat Oncol Biol Phys* **54**, 1537-1549 (2002).
- 135 Ivanov, S. *et al.* Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *Am J Pathol* **158**, 905-919 (2001).
- 136 Wykoff, C. C. *et al.* Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res* **60**, 7075-7083 (2000).
- 137 Olive, P. L. *et al.* Carbonic anhydrase 9 as an endogenous marker for hypoxic cells in cervical cancer. *Cancer Res* **61**, 8924-8929 (2001).
- 138 Lancaster, J. A. *et al.* Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res* **61**, 6394-6399 (2001).
- 139 Koukourakis, M. I. *et al.* Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. *Clin Cancer Res* **7**, 3399-3403 (2001).

- 140 Warburg, O. On the origin of cancer cells. *Science* **123**, 309-314 (1956).
- 141 Airley, R. E. *et al.* GLUT-1 and CAIX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding. *Int. J. Cancer* **104**, 85-91 (2003).
- 142 Mayer, A., Hockel, M., Wree, A. & Vaupel, P. Microregional expression of glucose transporter-1 and oxygenation status: lack of correlation in locally advanced cervical cancers. *Clin Cancer Res* **11**, 2768-2773 (2005).
- 143 Zhu, Y. *et al.* Hypoxia upregulates osteopontin expression in NIH-3T3 cells via a Ras-activated enhancer. *Oncogene* **24**, 6555-6563 (2005).
- 144 Le, Q. T. *et al.* Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. *Clin Cancer Res* **9**, 59-67 (2003).
- 145 Buffa, F. M., Harris, A. L., West, C. M. & Miller, C. J. Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. *Br J Cancer* **102**, 428-435 (2010).
- 146 Favaro, E., Lord, S., Harris, A. L. & Buffa, F. M. Gene expression and hypoxia in breast cancer. *Genome Medicine* **3** (2011).
- 147 Winter, S. C. *et al.* Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. *Cancer Res* **67**, 3441-3449 (2007).
- 148 Raleigh, J. A. *et al.* Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Res* **58**, 3765-3768 (1998).
- 149 Koch, C. J. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods Enzymol* **352**, 3-31 (2002).
- 150 Brown, J. M. & Le, Q. T. Tumor hypoxia is important in radiotherapy, but how should we measure it? *Int J Radiat Oncol Biol Phys* **54**, 1299-1301 (2002).
- 151 Kaanders, J. H. *et al.* Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. *Cancer Res* **62**, 7066-7074 (2002).
- 152 Horsman, M. R., Mortensen, L. S., Petersen, J. B., Busk, M. & Overgaard, J. Imaging hypoxia to improve radiotherapy outcome. *Nat Rev Clin Oncol* **9**, 674-87 (2012).
- 153 Koh, W. J. *et al.* Imaging of hypoxia in human tumors with [¹⁸F]-Fluoromisonidazole. *Int J Radiat Oncol Biol Phys* **22**, 199-212 (1992).
- 154 Eschmann, S. M. *et al.* Prognostic impact of hypoxia imaging with ¹⁸F-misonidazole PET in non-small cell lung cancer and head and neck cancer before radiotherapy. *J Nucl Med* **46**, 253-260 (2005).
- 155 Kawai, N. *et al.* Correlation of biological aggressiveness assessed by ¹¹C-methionine PET and hypoxic burden assessed by ¹⁸F-fluoromisonidazole PET in newly diagnosed glioblastoma. *Eur J Nucl Med Mol Imaging* **38**, 441-450 (2011).
- 156 Rajendran, J. G. *et al.* [¹⁸F]FMISO and [¹⁸F]FDG PET imaging in soft tissue sarcomas: Correlation of hypoxia, metabolism and VEGF expression. *Eur J Nucl Med Mol Imaging* **30**, 695-704 (2003).
- 157 Lawrentschuk, N. *et al.* Assessing regional hypoxia in human renal tumours using ¹⁸F-fluoromisonidazole positron emission tomography. *BJU Int* **96**, 540-546 (2005).
- 158 Hodgkiss, R. J. Use of 2-nitroimidazoles as bioreductive markers for tumour hypoxia. *Anticancer Drug Des* **13**, 687-702 (1998).
- 159 Chapman, J. D., Baer, K. & Lee, J. Characteristics of the metabolism-induced binding of misonidazole to hypoxic mammalian cells. *Cancer Res* **43**, 1523-1528 (1983).

- 160 Mannan, R. H. *et al.* Radioiodinated 1-(5-iodo-5-deoxy- β -D-arabinofuranosyl)-2-nitroimidazole (iodoazomycin arabinoside: IAZA): A novel marker of tissue hypoxia. *J Nucl Med* **32**, 1764-1770 (1991).
- 161 Casciari, J. J., Graham, M. M. & Rasey, J. S. A modeling approach for quantifying tumor hypoxia with [^{18} F]fluoromisonidazole PET time-activity data. *Med Phys* **22**, 1127-1139 (1995).
- 162 Rasey, J. S., Nelson, N. J., Chin, L., Evans, M. L. & Grunbaum, Z. Characteristics of the binding of labeled fluoromisonidazole in cells in vitro. *Rad Res* **122**, 301-308 (1990).
- 163 Bruehlmeier, M., Roelcke, U., Schubiger, P. A. & Ametamey, S. M. Assessment of hypoxia and perfusion in human brain tumors using PET with ^{18} F-fluoromisonidazole and ^{15}O -H $_2\text{O}$. *J Nucl Med* **45**, 1851-1859 (2004).
- 164 Burgman, P. *et al.* Cell line-dependent differences in uptake and retention of the hypoxia-selective nuclear imaging agent Cu-ATSM. *Nucl Med Biol* **32**, 623-630 (2005).
- 165 Mueller-Klieser, W. *et al.* Pathophysiological approaches to identifying tumor hypoxia in patients. *Radiother Oncol* **20**, 21-28 (1991).
- 166 Hlatky, L. & Alpen, E. L. Two-dimensional diffusion limited system for cell growth. *Cell Tissue Kinet* **18**, 597-611 (1985).
- 167 Dubois, L. *et al.* Evaluation of hypoxia in an experimental rat tumour model by [^{18} F]Fluoromisonidazole PET and immunohistochemistry. *Br J Cancer* **91**, 1947-1954 (2004).
- 168 Lee, N. Y. *et al.* Fluorine-18-Labeled Fluoromisonidazole Positron Emission and Computed Tomography-Guided Intensity-Modulated Radiotherapy for Head and Neck Cancer: A Feasibility Study. *Int J Radiat Oncol Biol Phys* **70**, 2-13 (2008).
- 169 Rasey, J. S. *et al.* Determining hypoxic fraction in a rat glioma by uptake of radiolabeled fluoromisonidazole. *Radiat Res* **153**, 84-92 (2000).
- 170 Gagel, B. *et al.* pO $_2$ polarography versus positron emission tomography ([^{18} F] fluoromisonidazole, [^{18} F]-2-fluoro-2'-deoxyglucose): An appraisal of radiotherapeutically relevant hypoxia. *Strahlenther Onkol* **180**, 616-622 (2004).
- 171 Zimny, M. *et al.* FDG - A marker of tumour hypoxia? A comparison with [^{18} F] fluoromisonidazole and pO $_2$ -polarography in metastatic head and neck cancer. *Eur J Nucl Med Mol Imaging* **33**, 1426-1431 (2006).
- 172 Gagel, B. *et al.* pO polarography, contrast enhanced color duplex sonography (CDS), [^{18} F] fluoromisonidazole and [^{18} F] fluorodeoxyglucose positron emission tomography: Validated methods for the evaluation of therapy-relevant tumor oxygenation or only bricks in the puzzle of tumor hypoxia? *BMC Cancer* **7** (2007).
- 173 Kubota, K. *et al.* Comparison of the distribution of fluorine-18 fluoromisonidazole, deoxyglucose and methionine in tumour tissue. *Eur. J Nucl Med* **26**, 750-757 (1999).
- 174 Bentzen, L. *et al.* Assessment of hypoxia in experimental mice tumours by [^{18} F]fluoromisonidazole PET and pO $_2$ electrode measurements - Influence of tumour volume and carbogen breathing. *Acta Oncol* **41**, 304-312 (2002).
- 175 Jansen, J. F. A. *et al.* Noninvasive assessment of tumor microenvironment using dynamic contrast-enhanced magnetic resonance imaging and ^{18} F-fluoromisonidazole positron emission tomography imaging in neck nodal metastases. *Int J Radiat Oncol Biol Phys* **77**, 1403-1410 (2010).

- 176 Rajendran, J. G. *et al.* Tumor hypoxia imaging with [F-18] fluoromisonidazole positron emission tomography in head and neck cancer. *Clin Cancer Res* **12**, 5435-5441 (2006).
- 177 Rasey, J. S. *et al.* Quantifying regional hypoxia in human tumors with positron emission tomography of [18F]fluoromisonidazole: A pretherapy study of 37 patients. *Int J Radiat Oncol Biol Phys* **36**, 417-428 (1996).
- 178 Thorwarth, D., Eschmann, S. M., Scheiderbauer, J., Paulsen, F. & Alber, M. Kinetic analysis of dynamic 18F-fluoromisonidazole PET correlates with radiation treatment outcome in head-and-neck cancer. *BMC Cancer* **5** (2005).
- 179 Wyss, M. T., Honer, M., Schubiger, P. A. & Ametamey, S. M. NanoPET imaging of [18F]fluoromisonidazole uptake in experimental mouse tumours. *Eur J Nucl Med Mol Imaging* **33**, 311-318 (2006).
- 180 Nehmeh, S. A. *et al.* Reproducibility of Intratumor Distribution of 18F-Fluoromisonidazole in Head and Neck Cancer. *Int J Radiat Oncol Biol Phys* **70**, 235-242 (2008).
- 181 Dirix, P. *et al.* Dose painting in radiotherapy for head and neck squamous cell carcinoma: Value of repeated functional imaging with 18F-FDG PET, 18F-fluoromisonidazole PET, diffusion-weighted MRI, and dynamic contrast-enhanced MRI. *J Nucl Med* **50**, 1020-1027 (2009).
- 182 Spence, A. M. *et al.* Regional hypoxia in glioblastoma multiforme quantified with [18F] fluoromisonidazole positron emission tomography before radiotherapy: Correlation with time to progression and survival. *Clin Cancer Res* **14**, 2623-2630 (2008).
- 183 Thorwarth, D., Eschmann, S. M., Holzner, F., Paulsen, F. & Alber, M. Combined uptake of [18F]FDG and [18F]FMISO correlates with radiation therapy outcome in head-and-neck cancer patients. *Radiother Oncol* **80**, 151-156 (2006).
- 184 Koh, W. J. *et al.* Evaluation of oxygenation status during fractionated radiotherapy in human nonsmall cell lung cancers using [F-18]fluoromisonidazole positron emission tomography. *Int J Radiat Oncol Biol Phys* **33**, 391-398 (1995).
- 185 Gupta, A. K., Cerniglia, G. J., Mick, R., McKenna, W. G. & Muschel, R. J. HIV protease inhibitors block Akt signaling and radiosensitize tumor cells both in vitro and in vivo. *Cancer Res* **65**, 8256-8265 (2005).
- 186 Brunner, T. B. *et al.* Phase I trial of the human immunodeficiency virus protease inhibitor nelfinavir and chemoradiation for locally advanced pancreatic cancer. *J Clin Oncol* **26**, 2699-2706 (2008).
- 187 Kelly, C. J. *et al.* Regulation of O2 consumption by the PI3K and mTOR pathways contributes to tumor hypoxia. *Radiother Oncol* **111**, 72-80 (2014).
- 188 Qayum, N. *et al.* Tumor vascular changes mediated by inhibition of oncogenic signaling. *Cancer Res* **69**, 6347-6354 (2009).
- 189 Fokas, E. *et al.* Dual inhibition of the PI3K/mTOR pathway increases tumor radiosensitivity by normalizing tumor vasculature. *Cancer Res* **72**, 239-248 (2012).
- 190 Xue, Q. *et al.* Palomid 529, a novel small-molecule drug, is a TORC1/TORC2 inhibitor that reduces tumor growth, tumor angiogenesis, and vascular permeability. *Cancer Res* **68**, 9551-9557 (2008).
- 191 Brunner, T. B. *et al.* Pancreatic cancer cell radiation survival and prenyltransferase inhibition: The role of K-ras. *Cancer Res* **65**, 8433-8441 (2005).

- 192 Cengel, K. A. *et al.* Oncogenic K-Ras signals through epidermal growth factor receptor and wild-type H-Ras to promote radiation survival in pancreatic and colorectal carcinoma cells. *Neoplasia* **9**, 341-348 (2007).
- 193 Gupta, A. K. *et al.* Radiation sensitization of human cancer cells in vivo by inhibiting the activity of PI3K using LY294002. *Int J Radiat Oncol Biol Phys* **56**, 846-853 (2003).
- 194 Zhang, H. *et al.* Locally advanced squamous cell carcinoma of the head and neck: CT texture and histogram analysis allow independent prediction of overall survival in patients treated with induction chemotherapy. *Radiology* **269**, 801-809 (2013).
- 195 Ganeshan, B., Panayiotou, E., Burnand, K., Dizdarevic, S. & Miles, K. Tumour heterogeneity in non-small cell lung carcinoma assessed by CT texture analysis: A potential marker of survival. *Eur Radiol* **22**, 796-802 (2012).
- 196 Mattonen, S. A., Palma, D. A., Haasbeek, C. J. A., Senan, S. & Ward, A. D. Early prediction of tumor recurrence based on CT texture changes after stereotactic ablative radiotherapy (SABR) for lung cancer. *Med Phys* **41**, 033502 (2014).
- 197 Varian Medical Systems. *Eclipse Treatment Planning System* Version 13.0. Available from www.varian.com
- 198 PMOD. *PMOD* Version 3.6. Available from www.pmod.com
- 199 IBM Corp. *IBM SPSS Statistics for Windows* Version 22.0. Available from www.ibm.com
- 200 Maitra, A. & Hruban, R. H. in *Annual Review of Pathology: Mechanisms of Disease* Vol. 3 157-188 (2008).
- 201 Tan, S. *et al.* Spatial-temporal [8F]FDG-PET features for predicting pathologic response of esophageal cancer to neoadjuvant chemoradiation therapy. *Int J Radiat Oncol Biol Phys* **85**, 1375-1382 (2013).
- 202 Rose, D. M. *et al.* 18Fluorodeoxyglucose-positron emission tomography in the management of patients with suspected pancreatic cancer. *Ann Surg* **229**, 729-738 (1999).
- 203 Wilson, J. M. *et al.* ARCI: A phase II trial of the HIV protease inhibitor Nelfinavir in combination with chemoradiation for locally advanced inoperable pancreatic cancer. *Radiother Oncol* **119**, 306-11 (2016).
- 204 Conroy, T. *et al.* FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. *N Engl J Med* **364**, 1817-1825 (2011).
- 205 Von Hoff, D. D. *et al.* Increased Survival in Pancreatic Cancer with nab-Paclitaxel plus Gemcitabine. *N Engl J Med* **369**, 1691-1703 (2013).
- 206 Humphris, J. L. *et al.* The prognostic and predictive value of serum CA19.9 in pancreatic cancer. *Ann Oncol* **23**, 1713-1722 (2012).
- 207 Koom, W. S., Seong, J., Kim, Y. B., Pyun, H. O. & Song, S. Y. CA 19-9 as a Predictor for Response and Survival in Advanced Pancreatic Cancer Patients Treated With Chemoradiotherapy. *Int J Radiat Oncol Biol Phys* **73**, 1148-1154(2009).
- 208 Delrue, L. *et al.* Assessment of tumor vascularization in pancreatic adenocarcinoma using 128-slice perfusion computed tomography imaging. *J Comput Assist Tomogr* **35**, 434-438 (2011).
- 209 Klauss, M. *et al.* Dual-energy perfusion-CT of pancreatic adenocarcinoma. *Eur J Radiol* **82**, 208-214 (2013).
- 210 Xu, J. *et al.* Pancreatic adenocarcinoma: dynamic 64-slice helical CT with perfusion imaging. *Abdom Imaging* **34**, 759-766 (2009).

- 211 Provenzano, P. P. & Hingorani, S. R. Hyaluronan, fluid pressure, and stromal resistance in pancreas cancer. *Br J Cancer* **108**, 1-8 (2013).
- 212 Olive, K. P. *et al.* Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* **324**, 1457-1461 (2009).
- 213 Zello, G. A., Smith, J. M., Pencharz, P. B. & Ball, R. O. Development of a heating device for sampling arterialized venous blood from a hand vein. *Ann Clin Biochem* **27**, 366-372 (1990).
- 214 Jensen, M. D. & Heiling, V. J. Heated hand vein blood is satisfactory for measurements during free fatty acid kinetic studies. *Metabolism* **40**, 406-409 (1991).
- 215 Abumrad, N. N., Rabin, D., Diamond, M. P. & Lacy, W. W. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism* **30**, 936-940, (1981).
- 216 Aerts, H. J. W. L. *et al.* Identification of residual metabolic-active areas within individual NSCLC tumours using a pre-radiotherapy 18Fluorodeoxyglucose-PET-CT scan. *Radiother Oncol* **91**, 386-392 (2009).
- 217 Hackett, S. L. *et al.* Estimation of input functions from dynamic [18F]FLT PET studies of the head and neck with correction for partial volume effects. *EJNMMI Research* **3**, doi: 10.1186/2191-219X-3-84 (2013).
- 218 Cobelli, C., Foster, D. & Toffollo, G. Tracer kinetics in biomedical research. *Kluwer Academic Publishers, New York*, 245-253, 262, 267 (2002).
- 219 Konishi, S. & Kitagawa, G. Information criteria and statistical modeling. *Springer, New York; London*, 29-104, 187-208 (2008).
- 220 Haber, E. & Modersitzki, J. Intensity gradient based registration and fusion of multi-modal images. *Medical image computing and computer-assisted intervention : MICCAI International Conference on Medical Image Computing and Computer-Assisted Intervention* **9**, 726-733 (2006).
- 221 Papiez, B. W., Heinrich, M. P., Fehrenbach, J., Risser, L. & Schnabel, J. A. An implicit sliding-motion preserving regularisation via bilateral filtering for deformable image registration. *Med Image Anal* **18**, 1299-1331 (2014).
- 222 South, C. P., Evans, P. M. & Partridge, M. Dose prescription complexity versus tumor control probability in biologically conformal radiotherapy. *Med Phys* **36**, 4379-4388 (2009).
- 223 Biehl, K. J. *et al.* 18F-FDG PET definition of gross tumor volume for radiotherapy of non-small cell lung cancer: Is a single standardized uptake value threshold approach appropriate? *J Nucl Med* **47**, 1808-1812 (2006).
- 224 Gooding, M. J. *et al.* Assessing The Quality of Deformable CT-MR Registration for the Purpose of Multimodal Radiotherapy Contouring. *Int J Radiat Oncol Biol Phys* **81**, S812-S813 (2011).
- 225 Lodge, M. *et al.* Combined PET/CT/MRI of the pelvis using software registration. *J Nucl Med Meeting Abstracts* **54**, 2089 (2013).
- 226 Fallone, B. G., Rivest, D. R. C., Riauka, T. A. & Murtha, A. D. Assessment of a commercially available automatic deformable registration system. *J Appl Clin Med Phys* **11**, 101-123 (2010).

- 227 Koong, A. C. *et al.* Pancreatic tumors show high levels of hypoxia. *Int J Rad Onc Biol Phys* **48**, 919-922 (2000).
- 228 Qin, R. *et al.* Combining clinicopathological predictors and molecular biomarkers in the oncogenic K-RAS/Ki67/HIF-1 α pathway to predict survival in resectable pancreatic cancer. *Br J Cancer* **112**, 514-522 (2015).
- 229 Whitfield, G. *et al.* Quantifying motion for pancreatic radiotherapy margin calculation. *Radiother Oncol* **103**, 360-366 (2012).
- 230 Liu, D., Chalkidou, A., Landau, D. B., Marsden, P. K. & Fenwick, J. D. 18F-FLT uptake kinetics in head and neck squamous cell carcinoma: A PET imaging study. *Med. Phys.* **41**, doi:10.1118/1.4868462 (2014).
- 231 Rasey, J. S. *et al.* Characterization of radiolabeled fluoromisonidazole as a probe for hypoxic cells. *Radiat Res* **111**, 292-304 (1987).
- 232 Ng, C. S. *et al.* Reproducibility of CT perfusion parameters in liver tumors and normal liver. *Radiology* **260**, 762-770 (2011).
- 233 Ben-Josef, E. *et al.* A phase I/II trial of intensity modulated radiation (IMRT) dose escalation with concurrent fixed-dose rate gemcitabine (FDR-G) in patients with unresectable pancreatic cancer. *Int J Radiat Oncol Biol Phys* **84**, 1166-1171 (2012).
- 234 Li, C. P. *et al.* Concurrent chemoradiotherapy treatment of locally advanced pancreatic cancer: Gemcitabine versus 5-fluorouracil, a randomized controlled study. *Int J Radiat Oncol Biol Phys* **57**, 98-104 (2003).
- 235 Chauffert, B. *et al.* Phase III trial comparing intensive induction chemoradiotherapy (60 Gy, infusional 5-FU and intermittent cisplatin) followed by maintenance gemcitabine with gemcitabine alone for locally advanced unresectable pancreatic cancer. Definitive results of the 2000-01 FFCD/SFRO study. *Ann Oncol* **19**, 1592-1599 (2008).
- 236 Topkan, E., Parlak, C., Kotek, A., Yapar, A. F. & Pehlivan, B. Predictive value of metabolic 18FDG-PET response on outcomes in patients with locally advanced pancreatic carcinoma treated with definitive concurrent chemoradiotherapy. *BMC Gastroenterol.* **11** (2011).
- 237 Michael Lovelock, D., Zatcky, J., Goodman, K. & Yamada, Y. The effectiveness of a pneumatic compression belt in reducing respiratory motion of abdominal tumors in patients undergoing stereotactic body radiotherapy. *Technology in Cancer Research and Treatment* **13**, 259-267(2014).
- 238 Huh, J. W., Park, Y. A., Jung, E. J., Lee, K. Y. & Sohn, S. K. Accuracy of endorectal ultrasonography and computed tomography for restaging rectal cancer after preoperative chemoradiation. *J Am Coll Surg.* **207**, 7-12 (2008).
- 239 Coroller, T. P. *et al.* CT-based radiomic signature predicts distant metastasis in lung adenocarcinoma. *Radiother. Oncol.* **114**, 345-50 (2014).
- 240 Lambin, P. *et al.* Radiomics: Extracting more information from medical images using advanced feature analysis. *Eur. J. Cancer* **48**, 441-446 (2012).
- 241 Kumar, V. *et al.* Radiomics: The process and the challenges. *Magn. Reson. Imaging* **30**, 1234-1248 (2012).
- 242 Ravanelli, M. *et al.* Texture analysis of advanced non-small cell lung cancer (NSCLC) on contrast-enhanced computed tomography: Prediction of the response to the first-line chemotherapy. *Eur Radiol* **23**, 3450-3455 (2013).

- 243 Ahmed, A., Gibbs, P., Pickles, M. & Turnbull, L. Texture analysis in assessment and prediction of chemotherapy response in breast cancer. *J Magn Reson Imaging* **38**, 89-101 (2013).
- 244 Li, J., Wilson JM & Partridge M. Texture Analysis Toolbox: a comprehensive analysis of molecular images for cancer research. *Medical Physics and Engineering Conference, Institute of Physics and Engineering in Medicine* (2014).
- 245 Chicklore, S. *et al.* Quantifying tumour heterogeneity in 18F-FDG PET/CT imaging by texture analysis. *Eur J Nucl Med Mol Imaging* **40**, 133-140 (2013).
- 246 Ganeshan, B., Miles, K. A., Young, R. C. D. & Chatwin, C. R. Texture analysis in non-contrast enhanced CT: Impact of malignancy on texture in apparently disease-free areas of the liver. *Eur J Radiol* **70**, 101-110 (2009).
- 247 El Naqa, I. *et al.* Exploring feature-based approaches in PET images for predicting cancer treatment outcomes. *Pattern recognition* **42**, 1162-1171 (2009).
- 248 Thibault, G., Fertil, B., Navarro, C. & Pereira, S. Texture indexes and gray level size zone matrix: application to cell nuclei classification. *Pattern Recognition Inf Process* 140-145 (2009).
- 249 Xu, D. H., Kurani, A. S., Furst, J. D. & Raicu, D. S. Run-length encoding for volumetric texture. *Proceedings of the Fourth IASTED International Conference on Visualization, Imaging, and Image Processing* 534-539 (2004).
- 250 Anantha, M., Moss, R. H. & Stoecker, W. V. Detection of pigment network in dermatoscopy images using texture analysis. *Computerized medical imaging and graphics : the official journal of the Computerized Medical Imaging Society* **28**, 225-234 (2004).
- 251 Amadasun, M. & King, R. Textural features corresponding to textural properties. *IEEE Transactions on Systems, Man, and Cybernetics* **19**, 1264-1274 (1989).
- 252 Aerts, H. J. W. L. *et al.* Decoding tumour phenotype by noninvasive imaging using a quantitative radiomics approach. *Nature Communications* **5**, doi: 10.1038/ncomms5006 (2014).
- 253 Levner, I. *et al.* Predicting MGMT methylation status of glioblastomas from MRI texture. *Medical image computing and computer-assisted intervention : MICCAI ... International Conference on Medical Image Computing and Computer-Assisted Intervention* **12**, 522-530 (2009).
- 254 Brown, R. *et al.* The use of magnetic resonance imaging to noninvasively detect genetic signatures in oligodendroglioma. *Clin Cancer Res* **14**, 2357-2362 (2008).
- 255 Dang, M. *et al.* MRI texture analysis predicts p53 status in head and neck squamous cell carcinoma. *Amer J Neuroradiol* **36**, 166-170 (2015).
- 256 Patel, U. B. *et al.* MRI after treatment of locally advanced rectal cancer: how to report tumor response--the MERCURY experience. *AJR* **199**, W486-495 (2012).
- 257 Haralick, R. M., Shanmugam, K. & Dinstein, I. Textural features for image classification. *IEEE Trans. Syst. Man Cybern.* **smc 3**, 610-621 (1973).
- 258 Leijenaar, R. T. H. *et al.* Stability of FDG-PET Radiomics features: An integrated analysis of test-retest and inter-observer variability. *Acta Oncol* **52**, 1391-1397 (2013).
- 259 Tixier, F. *et al.* Reproducibility of tumor uptake heterogeneity characterization through textural feature analysis in 18F-FDG PET. *J Nucl Med* **53**, 693-700 (2012).
- 260 Balagurunathan, Y. *et al.* Reproducibility and prognosis of quantitative features extracted from CT images. *Transl Oncol* **7**, 72-87 (2014).

- 261 Chen, W., Giger, M. L., Li, H., Bick, U. & Newstead, G. M. Volumetric texture analysis of breast lesions on contrast-enhanced magnetic resonance images. *Magn Reson Med* **58**, 562-571 (2007).
- 262 Ng, F., Kozarski, R., Ganeshan, B. & Goh, V. Assessment of tumor heterogeneity by CT texture analysis: Can the largest cross-sectional area be used as an alternative to whole tumor analysis? *Eur J Radiol* **82**, 342-348 (2013).
- 263 Win, T. *et al.* Tumor heterogeneity and permeability as measured on the CT component of PET/CT predict survival in patients with non-small cell lung cancer. *Clin Cancer Res* **19**, 3591-3599 (2013).
- 264 Schad, L. R. Problems in texture analysis with magnetic resonance imaging. *Dialogues Clin. Neurosci.* **6**, 235-242 (2004).
- 265 Enescu, M. *et al.* in *Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)* Vol. 8677 73-84 (2014).
- 266 Tofts, P. S. Modeling tracer kinetics in dynamic Gd-DTPA MR imaging. *J. Magn. Reson Imaging* **7**, 91-101 (1997).
- 267 Raleigh, J. A., Chou, S. C., Arteel, G. E. & Horsman, M. R. Comparisons among pimonidazole binding, oxygen electrode measurements, and radiation response in C3H mouse tumors. *Radiat Res.* **151**, 580-589 (1999).
- 268 Mikami, Y. *et al.* Carbonic anhydrase type IX expression in lobular endocervical glandular hyperplasia and gastric-type adenocarcinoma of the uterine cervix. *Pathology Research and Practice* **209**, 173-178 (2013).
- 269 Chalkley, H. Method for the quantitative morphological analysis of tissues. *J Natl Cancer Inst* **4**, 47-53 (1943).
- 270 Fox, S. B. *et al.* Quantitation and prognostic value of breast cancer angiogenesis: Comparison of microvessel density, Chalkley count, and computer image analysis. *J Pathol* **177**, 275-283 (1995).
- 271 Patel, U. B. *et al.* Magnetic resonance imaging-detected tumor response for locally advanced rectal cancer predicts survival outcomes: MERCURY experience. *J Clin Oncol* **29**, 3753-3760 (2011).
- 272 Goh, V. & Glynne-Jones, R. Perfusion CT imaging of colorectal cancer. *Br J Radiol* **87**, doi:10.1259/bjr.20130811 (2014).
- 273 Ng, C. S. *et al.* Reproducibility of perfusion parameters obtained from perfusion CT in lung tumors. *AJR* **197**, 113-121 (2011).
- 274 Loi, S. *et al.* Oxaliplatin combined with infusional 5-fluorouracil and concomitant radiotherapy in inoperable and metastatic rectal cancer: a phase I trial. *Br J Cancer* **92**, 655-661 (2005).
- 275 Bache, M. *et al.* Elevated tumor and serum levels of the hypoxia-associated protein osteopontin are associated with prognosis for soft tissue sarcoma patients. *BMC Cancer* **10**, doi:10.1186/1471-2407-10-132 (2010).
- 276 Dai, N., Bao, Q., Lu, A. & Li, J. Protein expression of osteopontin in tumor tissues is an independent prognostic indicator in gastric cancer. *Oncology* **72**, 89-96(2007).
- 277 Mack, P. C. *et al.* Lower osteopontin plasma levels are associated with superior outcomes in advanced non-small-cell lung cancer patients receiving platinum-based chemotherapy: SWOG study S0003. *J Clin Oncol* **26**, 4771-4776 (2008).
- 278 Maemura, K. *et al.* Role of positron emission tomography in decisions on treatment strategies for pancreatic cancer. *J Hepatobiliary Pancreat Surg* **13**, 435-441 (2006).

- 279 Bang, S. *et al.* The clinical usefulness of 18-fluorodeoxyglucose positron emission tomography in the differential diagnosis, staging, and response evaluation after concurrent chemoradiotherapy for pancreatic cancer. *J Clin Gastroenterol* **40**, 923-929 (2006).
- 280 Parlak, C., Topkan, E., Onal, C., Reyhan, M. & Selek, U. Prognostic value of gross tumor volume delineated by FDG-PET-CT based radiotherapy treatment planning in patients with locally advanced pancreatic cancer treated with chemoradiotherapy. *Radiation Oncology* **7** doi: 10.1186/1471-230X-11-123 (2012).
- 281 Devries, A. F. *et al.* Tumor microcirculation evaluated by dynamic magnetic resonance imaging predicts therapy outcome for primary rectal carcinoma. *Cancer Res* **61**, 2513-2516 (2001).
- 282 Bellomi, M., Petralia, G., Sonzogni, A., Zampino, M. G. & Rocca, A. CT perfusion for the monitoring of neoadjuvant chemotherapy and radiation therapy in rectal carcinoma: initial experience. *Radiology* **244**, 486-493 (2007).
- 283 Kremser, C., Trieb, T., Rudisch, A., Judmaier, W. & de Vries, A. Dynamic T(1) mapping predicts outcome of chemoradiation therapy in primary rectal carcinoma: sequence implementation and data analysis. *J Magn Reson Imaging* **26**, 662-671 (2007).
- 284 Lee, S. J. *et al.* Clinical implications of initial FDG-PET/CT in locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy. *Cancer Chemother. Pharmacol.* **71**, 1201-1207 (2013).
- 285 Sole, C. V. *et al.* Clinical significance of VEGFR-2 and 18F-FDG PET/CT SUVmax pretreatment score in predicting the long-term outcome of patients with locally advanced rectal cancer treated with neoadjuvant therapy. *Eur J Nucl Med Mol Imaging* **40**, 1635-1644 (2013).
- 286 Kremser, C. *et al.* Preliminary results on the influence of chemoradiation on apparent diffusion coefficients of primary rectal carcinoma measured by magnetic resonance imaging. *Strahlenther Onkol* **179**, 641-649 (2003).
- 287 Guerra, L. *et al.* Change in glucose metabolism measured by 18F-FDG PET/CT as a predictor of histopathologic response to neoadjuvant treatment in rectal cancer. *Abdom Imaging* **36**, 38-45 (2011).
- 288 Rosenberg, R. *et al.* The predictive value of metabolic response to preoperative radiochemotherapy in locally advanced rectal cancer measured by PET/CT. *Int J Colorectal Dis* **24**, 191-200 (2009).
- 289 Janssen, M. H. M. *et al.* Accurate Prediction of Pathological Rectal Tumor Response after Two Weeks of Preoperative Radiochemotherapy Using 18F-Fluorodeoxyglucose-Positron Emission Tomography-Computed Tomography Imaging. *Int J Radiat Oncol Biol Phys* **77**, 392-399 (2010).
- 290 Kim, Y. C. *et al.* Comparison of diffusion-weighted MRI and MR volumetry in the evaluation of early treatment outcomes after preoperative chemoradiotherapy for locally advanced rectal cancer. *J Magn Reson Imaging* **34**, 570-576 (2011).
- 291 Leibold, T. *et al.* Evaluation of 18F-FDG-PET for early detection of suboptimal response of rectal cancer to preoperative chemoradiotherapy: A prospective analysis. *Ann Surg Oncol* **18**, 2783-2789 (2011).
- 292 Goldberg, N. *et al.* Early prediction of histopathological response of rectal tumors after one week of preoperative radiochemotherapy using 18 F-FDG PET-CT imaging. A prospective clinical study. *Radiation Oncology* **7** (2012).

- 293 Janssen, M. H. *et al.* PET-based treatment response evaluation in rectal cancer: prediction and validation. *Int J Radiat Oncol Biol Phys* **82**, 871-876 (2012).
- 294 Lambrecht, M. *et al.* Value of diffusion-weighted magnetic resonance imaging for prediction and early assessment of response to neoadjuvant radiochemotherapy in rectal cancer: preliminary results. *Int J Radiat Oncol Biol Phys* **82**, 863-870 (2012).
- 295 Bampo, C. *et al.* Is the standardized uptake value of FDG-PET/CT predictive of pathological complete response in locally advanced rectal cancer treated with capecitabine-based neoadjuvant chemoradiation? *Oncology* **84**, 191-199 (2013).
- 296 Guillem, J. G. *et al.* Prospective assessment of primary rectal cancer response to preoperative radiation and chemotherapy using 18-fluorodeoxyglucose positron emission tomography. *Dis Colon Rectum* **43**, 18-24 (2000).
- 297 George, M. L. *et al.* Non-invasive methods of assessing angiogenesis and their value in predicting response to treatment in colorectal cancer. *Br J Surg* **88**, 1628-1636 (2001).
- 298 Calvo, F. A. *et al.* 18F-FDG positron emission tomography staging and restaging in rectal cancer treated with preoperative chemoradiation. *Int J Radiat Oncol Biol Phys* **58**, 528-535 (2004).
- 299 Capirci, C. *et al.* Sequential FDG-PET/CT reliably predicts response of locally advanced rectal cancer to neo-adjuvant chemo-radiation therapy. *Eur J Nucl Med Mol Imaging* **34**, 1583-1593 (2007).
- 300 Melton, G. B. *et al.* Efficacy of preoperative combined 18-fluorodeoxyglucose positron emission tomography and computed tomography for assessing primary rectal cancer response to neoadjuvant therapy. *J Gastrointest Surg* **11**, 961-969; discussion 969 (2007).
- 301 Vliegen, R. F. *et al.* Can an FDG-PET/CT predict tumor clearance of the mesorectal fascia after preoperative chemoradiation of locally advanced rectal cancer? *Strahlenther Onkol* **184**, 457-464 (2008).
- 302 Dinter, D. J. *et al.* Can dynamic MR imaging predict response in patients with rectal cancer undergoing cetuximab-based neoadjuvant chemoradiation? *Onkologie* **32**, 86-93 (2009).
- 303 Konski, A. *et al.* Use of Molecular Imaging to Predict Clinical Outcome in Patients With Rectal Cancer After Preoperative Chemotherapy and Radiation. *Int J Radiat Oncol Biol Phys* **74**, 55-59 (2009).
- 304 Calvo, F. A. *et al.* (18)F-FDG PET bio-metabolic monitoring of neoadjuvant therapy effects in rectal cancer: focus on nodal disease characteristics. *Radiother Oncol* **97**, 212-216 (2010).
- 305 Mak, D. *et al.* The use of PET in assessing tumor response after neoadjuvant chemoradiation for rectal cancer. *Radiother Oncol* **97**, 205-211 (2010).
- 306 Palma, P. *et al.* The value of metabolic imaging to predict tumour response after chemoradiation in locally advanced rectal cancer. *Radiation Oncology* **5**, 119 (2010).
- 307 Sun, Y. S. *et al.* Locally advanced rectal carcinoma treated with preoperative chemotherapy and radiation therapy: preliminary analysis of diffusion-weighted MR imaging for early detection of tumor histopathologic downstaging. *Radiology* **254**, 170-178 (2010).
- 308 Curvo-Semedo, L. *et al.* Rectal cancer: assessment of complete response to preoperative combined radiation therapy with chemotherapy--conventional MR volumetry versus diffusion-weighted MR imaging. *Radiology* **260**, 734-743 (2011).

- 309 Hur, H. *et al.* 18Fluoro-deoxy-glucose positron emission tomography in assessing tumor response to preoperative chemoradiation therapy for locally advanced rectal cancer. *J Surg Oncol* **103**, 17-24 (2011).
- 310 Kim, S. H., Lee, J. Y., Lee, J. M., Han, J. K. & Choi, B. I. Apparent diffusion coefficient for evaluating tumour response to neoadjuvant chemoradiation therapy for locally advanced rectal cancer. *Eur Radiol* **21**, 987-995 (2011).
- 311 Martoni, A. A. *et al.* Prospective study on the FDG-PET/CT predictive and prognostic values in patients treated with neoadjuvant chemoradiation therapy and radical surgery for locally advanced rectal cancer. *Ann Oncol* **22**, 650-656 (2011).
- 312 Chennupati, S. K. *et al.* Positron emission tomography for predicting pathologic response after neoadjuvant chemoradiotherapy for locally advanced rectal cancer. *American Journal of Clinical Oncology: Cancer Clinical Trials* **35**, 334-339 (2012).
- 313 Curvo-Semedo, L. *et al.* Usefulness of perfusion CT to assess response to neoadjuvant combined chemoradiotherapy in patients with locally advanced rectal cancer. *Acad Radiol* **19**, 203-213 (2012).
- 314 Engin, G. *et al.* Can diffusion-weighted MRI determine complete responders after neoadjuvant chemoradiation for locally advanced rectal cancer? *Diagnostic & Interventional Radiology* **18**, 574-581 (2012).
- 315 Huh, J. W., Min, J. J., Lee, J. H., Kim, H. R. & Kim, Y. J. The predictive role of sequential FDG-PET/CT in response of locally advanced rectal cancer to neoadjuvant chemoradiation. *Am. J Clin Oncol* **35**, 340-344 (2012).
- 316 Jung, S. H. *et al.* Predicting response to neoadjuvant chemoradiation therapy in locally advanced rectal cancer: diffusion-weighted 3 Tesla MR imaging. *J Magn Reson Imaging* **35**, 110-116 (2012).
- 317 Perez, R. O. *et al.* Accuracy of positron emission tomography/computed tomography and clinical assessment in the detection of complete rectal tumor regression after neoadjuvant chemoradiation: long-term results of a prospective trial (National Clinical Trial 00254683). *Cancer* **118**, 3501-3511 (2012).
- 318 Calvo, F. A. *et al.* 18F-FDG PET/CT-based treatment response evaluation in locally advanced rectal cancer: a prospective validation of long-term outcomes. *Eur J Nucl Med Mol Imaging* **40**, 657-667 (2013).
- 319 Genovesi, D. *et al.* Diffusion-weighted magnetic resonance for prediction of response after neoadjuvant chemoradiation therapy for locally advanced rectal cancer: preliminary results of a monoinstitutional prospective study. *Eur J Surg Oncol* **39**, 1071-1078 (2013).
- 320 Guillem, J. G. *et al.* Neither FDG-PET nor CT can distinguish between a pathological complete response and an incomplete response after neoadjuvant chemoradiation in locally advanced rectal cancer: A prospective study. *Ann Surg* **258**, 289-295 (2013).
- 321 Intven, M., Reerink, O. & Philipppens, M. E. Diffusion-weighted MRI in locally advanced rectal cancer : pathological response prediction after neo-adjuvant radiochemotherapy. *Strahlenther Onkol* **189**, 117-122 (2013).
- 322 Monguzzi, L. *et al.* Locally advanced rectal cancer: value of ADC mapping in prediction of tumor response to radiochemotherapy. *Eur J Radiol* **82**, 234-240 (2013).

- 323 Murcia Durendez, M. J. *et al.* The value of 18F-FDG PET/CT for assessing the response to neoadjuvant therapy in locally advanced rectal cancer. *Eur J Nucl Med Mol Imaging* **40**, 91-97 (2013).
- 324 Huh, J. W., Kwon, S. Y., Lee, J. H. & Kim, H. R. Comparison of restaging accuracy of repeat FDG-PET/CT with pelvic MRI after preoperative chemoradiation in patients with rectal cancer. *J Cancer Res Clin Oncol.* **141**, 353-359 (2015).
- 325 Dietz, D. W. *et al.* Tumor hypoxia detected by positron emission tomography with 60Cu-ATSM as a predictor of response and survival in patients undergoing Neoadjuvant chemoradiotherapy for rectal carcinoma: a pilot study. *Dis Colon Rectum* **51**, 1641-1648 (2008).
- 326 Guillem, J. G. *et al.* Sequential preoperative fluorodeoxyglucose-positron emission tomography assessment of response to preoperative chemoradiation: a means for determining longterm outcomes of rectal cancer. *J Am Coll Surg* **199**, 1-7 (2004).
- 327 Kalff, V., Duong, C., Drummond, E. G., Matthews, J. P. & Hicks, R. J. Findings on 18F-FDG PET scans after neoadjuvant chemoradiation provides prognostic stratification in patients with locally advanced rectal carcinoma subsequently treated by radical surgery. *J Nucl Med* **47**, 14-22 (2006).
- 328 Yeung, J. M. *et al.* Metabolic response of rectal cancer assessed by 18-FDG PET following chemoradiotherapy is prognostic for patient outcome. *Dis Colon Rectum* **54**, 518-525 (2011).
- 329 Ruby, J. A. *et al.* FDG-PET assessment of rectal cancer response to neoadjuvant chemoradiotherapy is not associated with long-term prognosis: a prospective evaluation. *Dis Colon Rectum* **55**, 378-386 (2012).
- 330 Capirci, C. *et al.* Long-term prognostic value of 18F-FDG PET in patients with locally advanced rectal cancer previously treated with neoadjuvant radiochemotherapy. *AJR. American Journal of Roentgenology* **187**, W202-208 (2006).
- 331 Orlhac, F. *et al.* Tumor texture analysis in 18F-FDG PET: Relationships between texture parameters, histogram indices, standardized uptake values, metabolic volumes, and total lesion glycolysis. *J Nucl Med* **55**, 414-422 (2014).
- 332 Tixier, F. *et al.* Intratumor heterogeneity characterized by textural features on baseline 18F-FDG PET images predicts response to concomitant radiochemotherapy in esophageal cancer. *J Nucl Med* **52**, 369-378 (2011).
- 333 Bundschuh, R. A. *et al.* Textural Parameters of Tumor Heterogeneity in (1)(8)F-FDG PET/CT for Therapy Response Assessment and Prognosis in Patients with Locally Advanced Rectal Cancer. *J Nucl Med* **55**, 891-897 (2014).
- 334 Gebejes, A. & Huertas, R. Texture Characterization based on Grey-Level Co-occurrence Matrix. *ICTIC* (2013).
- 335 Xiaoou, T. Texture information in run-length matrices. *IEEE Transactions on Image Processing* **7**, 1602-1609 (1998).
- 336 Sun, C. & Wee, W. G. Neighboring gray level dependence matrix for texture classification. *Computer Vision, Graphics and Image Processing* **23**, 341-352 (1983).