MAGNETIC RESONANCE IMAGING BIOMARKERS OF ANGIOGENESIS AND APOPTOSIS IN ADVANCED CANCER

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ABSTRACT

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This work explores the use of functional magnetic resonance imaging (MRI) scans to provide biomarkers of angiogenesis and of apoptosis in advanced colorectal and ovarian cancer. Dynamic contrast-enhanced MRI (DCE-MRI) and diffusion weighted MRI (DW-MRI), can be used to quantify changes in tumour microvasculature and tumour cellularity respectively and so have potential to provide biomarkers for use with molecularly-targeted agents. The ultimate aim is to use these biomarkers to accelerate clinical trials, to rationalise therapy and to improve patient outcomes.

The first paper in this thesis describes DCE-MRI and DW-MRI techniques used in combination with ELISA measurements of circulating markers of angiogenesis and cell death in metastatic colorectal cancer treated with conventional, oxaliplatin-based chemotherapy. The second project investigates the use of DW-MRI in the pre-clinical setting, using CRC xenografts that had been engineered to undergo synchronous caspase-driven apoptosis when exposed to doxycycline. The DW-MRI results were compared with circulating markers of cell death, measured by ELISA, and IHC markers of cell death. The final project evaluates DCE-MRI and DW-MRI parameter changes with cytotoxic chemotherapy with or without the VEGF-inhibitor cediranib in platinum-sensitive advanced ovarian cancer. MRI parameters are compared with progression free and overall survival data to assess for prognostic biomarkers.

Both clinical studies demonstrate significant early change in MRI parameters following two cycles of treatment, reflecting treatment-induced alterations to the tumour size, microvasculature and cellularity. Following two cycles of chemotherapy, both studies revealed correlations between MRI parameters and survival. In the case of ovarian cancer, pre-treatment DCE-MRI markers were prognostic, regardless of treatment arm. In the pre-clinical setting, we have shown that DW-MRI can detect apoptosis in an *in vivo* model. Changes observed on DW-MRI reflected those changes observed using immunohistochemical and circulating marker analyses. The use of this highly controllable model provides a better understanding of the mechanisms and timing behind ADC alterations due to apoptosis. Taken together, these studies show that these DCE-MRI and DW-MRI techniques are feasible and safe in the pre-clinical and clinical settings and that they can provide clinically useful information that relates to outcome.

This thesis is presented in an alternative format to incorporate the three separate studies that were carried out to explore this common theme. The thesis is constructed around these three studies, which are written as journal papers.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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THE AUTHOR

Dr Danielle Shaw works as a Registrar in Medical Oncology and carried out the work contained in this thesis during three years 'out of program' from Specialist Training. This research was carried out between CRUK: Manchester Institute; The Wolfson Molecular Imaging Centre, Manchester; The Imaging Sciences Department, University of Manchester and The Christie Hospital NHS Foundation Trust. This work was funded and supported by Cancer Research UK and AstraZeneca Pharmaceuticals, as part of their CRUK-AZ Clinical Fellowships Programme.

ABBREVIATION LIST

ADC	Apparent diffusion coefficient
Ang	Angiopoietin
AUC	Area under the (time-contrast agent) curve
BRCA	Breast cancer associated gene
CA125	Cancer antigen 125
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CRC	Colorectal cancer
CRUK	Cancer Research UK
CT	Computerised tomography
CTC	Circulating tumour cells
CTCAE	Common Toxicity Criteria Adverse Events
DCE-MRI	Dynamic contrast enhanced magnetic resonance imaging
DW-MRI	Diffusion weighted magnetic resonance imaging
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbent assay
5-FU	5-Fluorouracil
FA	Folinic acid
FGF	Fibroblast growth factor
FOLFIRI	5-FU, FA and irinotecan
FOLFOX	5-FU, FA and oxaliplatin
GCSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumour
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary nonpolyposis colorectal cancer
ICON	International Collaborative Ovarian Neoplasm
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene
K^{trans}	Transfer constant reflecting contrast delivery and endothelial permeability.
MRI	Magnetic resonance imaging
OS	Overall survival
PDGF	Platelet derived growth factor
PlGF	Placental growth factor
PFS	Progression free survival
PPE	Palmar-plantar erythema
RECIST	Response Evaluation Criteria in Solid Tumors
RR	Response rate
v_e	The volume of extravascular extracellular space per unit volume of tissue.
v_p	The volume of plasma per unit volume of tissue
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WTV	Whole tumour volume
XELOX	Capecitabine and oxaliplatin

OVERVIEW OF THESIS

Background

This work explores the use of functional MRI to provide biomarkers of angiogenesis and of apoptosis in advanced colorectal and ovarian cancer. Recent efforts in cancer research have focused on the development of novel agents, which target biological pathways, such as those that regulate angiogenesis and apoptosis. Dynamic contrast-enhanced MRI and diffusion weighted MRI, can be used to quantify changes in tumour microvasculature and tumour cellularity and so have potential to provide biomarkers for use with these novel agents. Validation of the MRI parameters as biomarkers and as surrogate endpoints is needed before they can be confidently relied upon to inform clinical and trial design decisions. Ultimately, the aim is to use these biomarkers to accelerate clinical trials, to rationalise therapy and to improve patient outcomes.

Method

The first study in this thesis used DCE-MRI and DW-MRI techniques in combination with ELISA measurements of circulating markers of angiogenesis and cell death in metastatic colorectal cancer treated with conventional, oxaliplatin-based chemotherapy. As an exploratory aim, we measured circulating tumour cells and compared these results with changes in tumour microvasculature, derived from DCE-MRI.

The second project investigated the use of DW-MRI in the pre-clinical setting, using CRC xenografts that had been engineered to undergo synchronous caspase-driven apoptosis when exposed to doxycycline. The DW-MRI results were compared with circulating markers of cell death, measured by ELISA, and IHC markers of cell death.

The final project was a DCE-MRI and DW-MRI imaging sub-study to the phase III MRC ICON6 study using chemotherapy with or without the VEGF inhibitor cediranib in platinum-sensitive advanced ovarian cancer. MRI parameters were compared with progression free and overall survival data to assess for prognostic biomarkers.

Results

Results of project one showed that the MRI-determined whole tumour volume (WTV), fell significantly in all colorectal liver metastases by chemotherapy cycle 2 day 2 (C2D2) and reduced further in all tumours by cycle 6. By C2D2, the cohort DCE-MRI-derived iAUC, K^{trans} and v_e were significantly higher than before treatment and remained elevated after cycle 6. Patients with a K^{trans} higher than pre-treatment levels at C2D2 had an overall survival of 10.1 months whereas the equivalent statistic was 22.1 months in those without a significant rise at this time-point ($p = 0.049$). A positive association existed between MRI derived WTV and CTC number at the pre-treatment time-point ($p = 0.005$). A negative correlation was found between K^{trans} and CTC number before treatment ($p = 0.042$) and at C1D2 time-point ($p= 0.018$). The median apparent diffusion coefficient (ADC) in this cohort significantly increased by C2D2 ($p = 0.004$) and C6 ($p = 0.005$) but ADC change did not relate to survival.

In the preclinical setting, project two found that DW-MRI, together with immunohistochemical (IHC) analysis of tumour tissue and measurement of circulating biomarkers of apoptosis, detected apoptosis within 6 hours of induction of the process. The median ADC was significantly increased in tumours that had been modified to undergo synchronous caspase driven apoptosis ('death switched') 6 hours following induction of the death switch (mean change in ADC 0.041 x $10^{-3}mm^2/s$, $p=0.034$). No significant change was seen in the median ADC in the control tumours (mean change in ADC 0.014 x $10^{-3}mm^2/s$, $p=0.447$). No difference in median ADC was observed in either cohort at 24 hours post death switch induction. There were significant increases in the plasma concentrations of cleaved CK18 in the death switched tumours but not in control tumours at 6 and 24 hours after induction with doxycycline, indicating significant apoptosis in tumours at these time points (6 hours: death switched mean 661U/L, control mean 311 U/L, $p = 0.046$; 24 hours: death switched mean 947 U/L, control mean 238 U/L, $p < 0.0001$).

Project three revealed potential prognostic biomarkers in patients with advanced ovarian cancer. Those patients who had a significant reduction in WTV by C2D2 had a significantly longer PFS and OS than those who did not (PFS: 10.2 versus 7.7 months, $p = 0.046$; OS: 34.6 versus 19.4 months, $p = 0.006$). Pre-treatment $IAUC_{60}$ demonstrated a

significant negative correlation with a prolonged PFS and OS (PFS: r = -0.73, p = 0.007; OS: r = -0.61, p = 0.034). A low pre-treatment K^{trans} was associated with an improved PFS and OS (PFS: r = -0.67, p = 0.017; OS: r = -0.70, p = 0.012). The DW-MRI parameter ADC was not altered significantly by chemotherapy or by chemotherapy plus cediranib. However, an increase in ADC at C1D2 did correlate significantly with an increase in PFS (r = 0.63, p = 0.028).

Conclusion

Both clinical studies demonstrated significant early change in MRI parameters following two cycles of treatment, reflecting treatment-induced alterations to the tumour size, microvasculature and cellularity. By C2D2, both studies revealed correlations between MRI parameters and outcome. In the case of ovarian cancer, pre-treatment DCE-MRI markers were prognostic, regardless of treatment arm.

In the pre-clinical setting, DW-MRI can detect apoptosis in an in vivo model. Changes observed on DW-MRI largely reflected those changes observed using immunohistochemical and circulating marker analyses. The use of this highly controllable model provides a better understanding of the mechanisms and timing behind ADC alterations due to apoptosis.

Taken together, these studies show that these DCE-MRI and DW-MRI techniques are feasible and safe in the pre-clinical and clinical settings and that they can provide clinically useful information that relates to outcome. Further work should seek to expand beyond these small studies to allow the potential biomarkers we have identified to be validated for use in clinical trials.

INTRODUCTION

Despite improvements in cytotoxic cancer treatment in recent years, survival rates for many solid cancers remain poor (Quaresma et al 2015). The increase of doublet to triplet cytotoxic chemotherapy regimens has been associated with significant toxicity with modest benefit in most cases. With an ever increasing understanding of cancer biology, potential drug targets have been identified in many areas including angiogenesis and apoptosis. Recent efforts in cancer research have focused on the development of novel agents, which target these pathways (Jayson et al 2016; Letai 2008). Biomarkers, such as those provided by functional MRI techniques described in this thesis, can be used to accelerate the development of these drugs by providing, for instance, critical information on proof of principle.

The development of these mechanistically targeted agents poses challenges to traditional models of drug development. Therapies targeting the angiogenic and apoptotic pathways may show cytostatic rather than cytotoxic results meaning that evaluation of radiological response rates, for example with RECIST criteria, may be inadequate (Ratain and Eckhardt 2004; Eisenhauer 2009). Novel agents may not show an acute dose-limiting toxicity, the end-point used to define maximum tolerated dose in phase I dose finding studies using cytotoxic therapies. Thus alternative approaches to drug development are needed. The Food and Drug Administration summarised the roles of biomarkers and surrogate endpoints as providing evidence for the validation of therapeutic targets; the selection of mechanism of action of drug candidates; the demonstration of proof-of-principle for therapeutic interventions; the identification of patient sub-populations and the evaluation of response to therapy (FDA report 2004). It is clear that there is an urgent need to identify and validate suitable markers to streamline clinical trials, rationalise therapy and ultimately to improve patient outcomes.

Imaging biomarkers have a number of advantages. They are non-invasive and so can be used on multiple occasions throughout the course of the disease. Imaging results can be quantified and modelled and so are versatile, allowing for multiple different parameters to be determined from one data set. Functional imaging can provide cellular and molecular information rather than focusing solely on size changes. An example of this would be the use of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) parameters to assess changes in tumour microvasculature with the use of vascular endothelial growth

factor (VEGF) receptor inhibiting drugs. There are currently several challenges to overcome in the application of functional imaging biomarkers. Standardisation of the techniques and terms used is necessary if multi-centre trials are to become feasible. Validation of the parameters as biomarkers and, ultimately, as surrogate endpoints is needed before they can be confidently relied upon to inform clinical and trial design decisions.

Dynamic contrast-enhanced MRI (DCE-MRI) techniques have been used extensively in early phase clinical trials of drugs targeting the VEGF pathway. The parameters derived from these images give information regarding the dynamic changes in the tumour microvasculature associated with inhibition of VEGF (O'Connor et al 2012). In this thesis, the focus is on the use of functional MRI parameters as biomarkers in metastatic colorectal cancer and relapsed ovarian cancer. Angiogenesis is believed to play a key role in the pathogenesis of both of these diseases. This, and the high mortality associated with these malignancies, has led to an interest in the use of anti-angiogenic drugs in these settings.

Diffusion weighted MRI (DW-MRI) is a newer technique that can be used to characterise the cellularity and extravascular space in a tissue. The derived parameter of DW-MRI, the apparent diffusion coefficient (ADC), has potential as a marker of apoptosis in tumour cells (Koh and Collins 2007). Apoptotic pathways are now well characterised, with defective pathways contributing to resistance to conventional chemotherapies. The development of drugs to target these defective apoptotic pathways is currently underway, leading to an increasing need for markers of tumour tissue apoptosis.

The first study in this thesis explores the use of DCE-MRI and DW-MRI techniques in combination with circulating markers of angiogenesis and cell death in metastatic colorectal cancer (mCRC) treated with conventional, oxaliplatin-based chemotherapy. The second project investigated the use of DW-MRI in the pre-clinical setting, using CRC xenografts that had been engineered to undergo synchronous caspase driven apoptosis when exposed to doxycycline. The final project is a DCE-MR and DW-MR imaging sub-study to the phase III MRC ICON6 study using chemotherapy with or without the VEGF inhibitor cediranib in platinum-sensitive advanced ovarian cancer.

The long-term aim of these functional imaging studies is to develop robust biomarkers to predict early response to both conventional and targeted agents to allow for timely management decisions, which would ultimately benefit the patient.

ANGIOGENESIS

Drugs targeting the angiogenic pathways are of current interest in the treatment of both advanced colorectal and advanced ovarian cancers (Tampellini et al 2016; Petrillo et al 2016). Biomarkers that predict benefit from anti-angiogenic agents are limited but are required to support drug development and to guide treatment.

The term angiogenesis refers to the process by which new blood vessels are formed from pre-existing capillaries. This differs from vasculogenesis, which describes the formation of new endothelial cells from precursor cells in the embryo or bone marrow. Angiogenesis is a multi-step process that begins with detachment of pericytes and blood vessel dilatation, followed by degeneration of the basement membrane surrounding the endothelial cells. This allows endothelial cells to migrate into the perivascular space towards angiogenic stimuli and to proliferate, forming endothelial columns. These proliferating cells adhere to each other and eventually form the lumen of a new capillary. Finally, the new vessels fuse with other sprouts to form a new vascular system. This process is largely restricted to three physiological processes: menstruation, embryonic growth and wound healing.

Solid tumours secrete angiogenic factors, which lead to angiogenesis within the tumour (Folkman 1971). Without a new vasculature, the tumour will not have the necessary oxygen and nutritional input to grow beyond $1mm^3$. Sustained angiogenesis was named as one of the 'hallmarks of cancer' in the seminal paper by Hanahan and Weinberg 2000. It is the observed difference between sustained angiogenesis in tumours and the low activity in healthy adult tissues that make inhibition of angiogenesis such an attractive therapeutic strategy.

Regulation of Angiogenesis

Hanahan and Folkman hypothesised that pro- and anti-angiogenic factors are tightly regulated in healthy tissues and that it is loss of this balance which leads to the acquisition of the angiogenic phenotype in tumours, the so called 'angiogenic switch'. These factors are secreted by tumour cells and host infiltrating cells, such as macrophages. In preclinical models, the angiogenic switch can be induced at different stages in tumour growth, depending on environmental and genetic factors. Environmental changes resulting in the induction of the angiogenic switch include hypoxia, pH alterations, metabolic stress and inflammatory cytokine release (Scweiki 1992). Oncogene activation such as Src or Ras can

trigger angiogenesis whereas activation of the tumour-suppressors p53 and von Hippel-Lindau may downregulate the process (see *table I1*).

PRO-ANGIOGENIC FACTORS	ANTI-ANGIOGENIC FACTORS
Growth factors	Angiostatin
VEGF	Endostatin
FGF	**Cytokines**
PlGF	Interleukin -4,-10, -12 and -18
GCSF	Interferon –α and –β
Cytokines	**Soluble factors**
Interleukin-1, -6, -8	FGFR-1
Oncogenes	VEGFR-1
Bcl-2	**Vitamins**
EGFR	Vitamin D3
HER2	Retinoic acid
KRAS	**Tumour suppressor genes**
Others	p53
Hypoxia	p16
Angiopoietin-1	
Erythropoetin	

Table I1: Selected pro- and anti-angiogenic factors. VEGF: Vascular endothelial growth factor, FGF: Fibroblast growth factor, PlGF: Placental growth factor, GCSF Granulocyte colony stimulating factor, Bcl-2: B-cell lymphoma 2, EGFR Epithelial Growth Factor, HER2: human epidermal growth factor receptor 2, KRAS: Kirsten rat sarcoma viral oncogene, FGFR-1: Fibroblast growth factor receptor 1.

Vascular Endothelial Growth Factors

The vascular endothelial growth factors and their receptors play a key role in both physiological and pathological angiogenesis, making it an attractive target for targeted therapies. The VEGF family is made up of at least seven members: VEGF-A to E and the placental growth factors, PlGF-1 and 2. VEGF-A is the most clearly defined member of the VEGF family and is commonly referred to as 'VEGF.' It is believed to be a vital mediator of tumour angiogenesis. It is secreted from most tumour cells and exists in at least four isoforms; VEGF-A_{165} being most commonly expressed in tumours (Ferrara et al 2003).

Other isoforms differ in their affinity for heparin and may play distinct roles in angiogenesis (Kowanetz et al 2006). The protein now known as VEGF was initially referred to as 'vascular permeability factor' (VPF) due to its ability to induce vascular permeability in tumours that cause ascitic fluid to accumulate (Senger et al 1983). Independently of this work, several groups isolated VEGF protein, which displayed the same actions as VPF and it is now known that these two factors are, in fact, the same. It is now known that binding of VEGF to the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) promotes angiogenesis by activation of endothelial cells, promotion of cell survival signals, increased invasion and migration as well as by increasing vascular permeability (Hicklin and Ellis 2005) (see *table I2*).

GROWTH FACTORS	**CELL MEMBRANE RECEPTOR**
VEGF-A_{121} VEGF-A_{165} VEGF-B PlGF	VEGFR-1 (flt-1)
VEGF-A_{121} VEGF-A_{165} VEGF-C	VEGFR-2 (flk-1/KDR)
VEGF-C	VEGFR-3 (flt-3)

Table I2: VEGF family growth factors and their associated receptors.

ANGIOGENESIS IN METASTATIC COLORECTAL CANCER

Colorectal cancer epidemiology

Colorectal carcinoma (CRC) is common in the United Kingdom, with around 34,000 newly diagnosed cases in England each year (Cancer Registration Statistics, England 2014). Despite improvements in treatment strategies, five-year overall survival remains around 58% (Cancer Survival in England, 2015). Approximately 30% of those diagnosed with colorectal cancer present with advanced disease and the five-year survival is then poor, at approximately 10%. The vast majority of cases of colorectal cancer are sporadic with familial syndromes, such as familal adenomatous polyposis and hereditary non-polyposis colorectal cancer making up a minority of cases. An increasing number of lifestyle factors have now been shown to increase risk, including smoking, high red meat consumption, low fibre intake, excess alcohol use and obesity (Romaguera et al 2015).

Staging of colorectal cancer

Colorectal cancer was previously staged using a modified version of the Duke's system, devised in 1932 (Dukes CE and Bussey HJ, 1932). This has now been largely superseded by the TNM classification system, in line with many other tumour groups (Sobin et al 2009). The focus of my studies will be on those patients in whom distant metastases are present and in whom survival is currently poor.

Surgical management of metastatic colorectal cancer

For patients with distant metastases, resection of the metastases is the only potentially curative treatment option. However, up to 80% of patients with liver metastases at disease presentation are considered unsuitable for metastasectomy (Poston et al 2008). A highly selected group of patients with pulmonary metastases may be offered resection but this group currently remains the minority (Pfannschmidt et al 2007). Although some metastatic lesions may become resectable with the use of neoadjuvant chemotherapy, many will not. Palliative chemotherapy remains the mainstay of treatment with the aim to prolong life, lessen symptoms and improve quality of life.

Cytotoxic management of metastatic colorectal cancer

5-Fluorouracil

5-Flurouracil (5-FU) has formed the basis of the cytotoxic management of colorectal cancer for many years (Heidelberger et al 1957). 5-FU exerts its cytotoxic effects through

various pathways, including inhibition of thymidylate synthase. This inhibition leads to depletion of deoxythymidine triphosphate and so interferes with DNA synthesis and repair. 5-FU is also incorporated into RNA, resulting in alterations in RNA processing and into DNA, resulting in inhibition of DNA synthesis and function. 5-FU based chemotherapy confers an overall survival benefit over supportive care alone (Simmonds 2000) and can improve quality of life when used in the palliative setting. It is used in combination with the modulating agent folinic acid, which potentiates the inhibitory effect on thymidylate synthase and so increases the cytotoxic activity of 5-FU. This improves response rates and survival in the palliative setting (Thirion et al 2004).

Capecitabine

Capecitabine is an oral pro-drug, which is converted to 5-FU. It is converted preferentially at the tumour site, due to higher levels of thymidine phosphorylase in tumour cells than in normal cells. Capecitabine has been found to have at least equivalent efficacy to 5-FU in the treatment of metastatic colorectal cancer (Hoff et al 2001; Van Cutsem et al 2001) and subsequent trials have confirmed that the combination of oxaliplatin with capecitabine (XELOX) is non-inferior to oxaliplatin with 5-FU (FOLFOX) (Arkenau et al 2008). Capecitabine is therefore now used as part of the conventional cytotoxic management of metastatic colorectal cancer in the UK.

Oxaliplatin

Like other platinum compounds, oxaliplatin acts through formation of platinum-DNA adducts, which cross-link the DNA double-helix and exert a cytotoxic effect by blocking DNA replication and transcription. Oxaliplatin works synergistically with 5-FU and the combination of the two agents has been evaluated in several large phase III trials (Giacchetti et al 2000. De Gramont et al 2000; Grothey et al 2002). Although these trials have not shown an overall survival benefit attributable to the addition of oxaliplatin to 5-FU, they have found improved response rates and longer progression free survival. The addition of oxaliplatin to 5-FU regimens is now considered standard treatment for advanced colorectal cancer in the first line and subsequent settings.

Irinotecan

Irinotecan is a pro-drug of SN-38, which exerts its effect by inhibition of topoisomerase I (Topo1). SN-38 binds to the Topo1-DNA complex, causing the accumulation of DNA

cleavable complexes and leading to cytotoxic DNA damage in replicating cells. Several large trials have shown efficacy of irinotecan in combination with 5FU/folinic acid (Douillard et al 2000; Saltz et al 2000; Kohne et al 2005) and it is now considered a first line treatment option in the palliation of metastatic colorectal cancer.

Chemotherapy is given empirically in metastatic colorectal cancer and it is clear that response rates vary widely. With the newer, targeted therapies, predictive biomarkers are increasingly shifting management strategies towards tumour biology-focused treatments. An example of this is the routine use of tumour KRAS testing in the use of epidermal growth factor receptor inhibitors (Karapetis et al 2008). The angiogenic and apoptotic pathways are thought to be crucial for progression of tumours (Hanahan and Weinberg 2000) and both may be targeted in the treatment of colorectal cancer, as described below.

Angiogenesis in metastatic CRC

VEGF levels are significantly higher in the serum and plasma of patients with colorectal cancer (CRC) compared with healthy volunteers (Kut et al 2007). Interestingly, the timing of the VEGF angiogenic switch during CRC progression has been studied by measuring the gene expression of VEGF ligands (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) and their receptors (VEGFR-1, VEGFR-2, and VEGFR-3) in colorectal tissue, adenomas and in CRC at all Dukes stages. They found significantly higher levels of VEGF-A and VEGF-B mRNAs in adenomas compared with normal tissues and increased VEGF-A and VEGF-C in carcinomas compared with normal tissues. However, there was a significant reduction of VEGF-B in carcinomas compared with adenomas. VEGFR-1 was significantly correlated with tumour grade, Dukes stage, and lymph node involvement; VEGFR-2 with lymph node involvement alone and VEGFR-3 did not correlate with any of the variables tested. They concluded that VEGF-A and VEGF-B play a role in adenoma formation and that VEGF-C is important in carcinoma formation (Hanrahan et al 2003). Tumour PlGF levels have also been shown to be high and significantly correlated with disease progression and overall survival in colorectal cancer (Wei et al 2005). These findings support the hypothesis that angiogenesis plays a crucial role in the pathophysiology of colorectal cancer.

VEGF inhibition in metastatic colorectal cancer (mCRC)

Bevacizumab

Bevacizumab is a humanised monoclonal antibody that targets the biological activity of VEGF-A. Preclinical development of bevacizumab revealed a direct anti-angiogenic effect, leading to regression of tumour xenografts (Presta et al 1997). In addition it may also act to improve the delivery of chemotherapy by altering tumour vasculature and by decreasing the pathologically elevated interstitial pressure in tumours (Jain 2001; Willett et al 2004). Preclinical data have suggested that tumour vasculature normalises in vivo after treatment with VEGF inhibitors, leading to decreased vascular permeability and increased pericyte coverage. This VEGF inhibitor induced normalisation leads to an improved response to radiation in the preclinical setting, due to improved perfusion and tumour oxygenation (Winkler et al 2004; Batchelor et al 2007). Initial reports of clinical efficacy came from a large (n = 813) randomised phase III trial, which reported a statistically significant difference in median overall survival (OS) when 5mg/kg/2 weeks bevacizumab was added to first-line chemotherapy in metastatic CRC compared with irinotecan/5-FU/leucovorin alone using a bolus IFL regimen (*table I3*) (20.3 v 15.6 months, HR 0.66, 95% confidence interval 0.54 to 0.81) (Hurwitz et al 2004). However, the addition of bevacizumab to first-line FOLFIRI (*table I3*) has not shown a survival benefit in two phase III trials (Stathopoulos et al 2010; Passardi et al 2015). A phase III study of bevacizumab in combination with first-line FOLFOX4 or XELOX (*table I3*) showed a significant improvement in progression free survival (PFS) but not in OS (21.3 v 19.9 months, HR 0.89, 97.5% CI 0.76 to 1.03) (Saltz et al 2008). This may be related to the finding that only 29% of the patients in the chemotherapy plus bevacizumab arm were treated until disease progression, in contrast with previous studies, or because the cytotoxic component in the latter trials was more effective. A recent systematic review of randomised trials in first and second line treatment of metastatic CRC concluded that the addition of bevacizumab to chemotherapy significantly improved progression free and overall survival by approximately 2 months (PFS 9.1 versus 6.9 months, HR 0.58, 95% CI 0.46-0.73; OS 19.8 versus 17.6 months, HR 0.80, 95% CI 0.71-0.90) (Hurwitz et al 2013). This is at the expense of higher grade toxicities including bleeding, proteinuria, hypertension, thromboembolic events and bowel perforations, which although uncommon, are clearly potentially serious. Bevacizumab has now been licensed for first line use in combination with cytotoxic chemotherapy in metastatic colorectal cancer.

Modest improvements in OS were seen in a randomised trial of bevacizumab in combination with FOLFOX4 in the second line setting, with a statistically significant improvement in OS from 10.8 to 12.9 months (HR 12.9, p= 0.001) with the addition of bevacizumab (Giantonio et al 2007). An association between post-progression bevacizumab therapy and prolonged survival has also been suggested by a large, prospective, observational study (Grothey et al 2013). This issue has subsequently been addressed in two phase III trials; TML and BBEBYP. In the TML study, 820 patients with unresectable mCRC progressing within 3 months of receiving first-line chemotherapy including bevacizumab were randomised to fluoropyrimidine-containing chemotherapy with or without bevacizumab. Bevacizumab significantly improved OS (11.2 versus 9.8 months HR 0.81, 95% CI 0.69-0.94) (Bennouna et al 2013). The BEBYP trial randomised 185 patients undergoing first-line chemotherapy with bevacizumab to receive second-line FOLFOX or FOLFIRI with or without bevacizumab but the trial was stopped prematurely when the results of the TML study became available. Even so, PFS was found to be significantly improved by second-line bevacizumab (6.8 versus 5.0 months, HR 0.70, 95% CI 0.52 to 0.95) (Masi et al 2015). Bevacizumab is not currently recommended beyond first progression in mCRC.

Regimen	Irinotecan	Oxaliplatin	Folinic acid	5-FU/ Capecitabine	Schedule
IFL	125mg/m^2	-	20 mg/m^2	5FU 500 mg/m^2	Once weekly for 4 wk; 6 wk cycle
FOLFIRI	135 mg/m^2	-	200mg/m^2	5FU 500 mg/m^2	3 week cycle
FOLFOX4	-	85mg/m^2	400 mg/m^2	5FU 400mg/m^2 bolus plus 600 mg/m^2 over 22 hours day 1 and day 2	2 week cycle
FOLFOX6	-	100 mg/m²	400 mg/m²	5FU 400 mg/m² bolus, plus 2400 mg/m² over 46 hours	2 week cycle
XELOX	-	130mg/m^2	-	Capecitabine 1000 mg/m^2 bd	Capecitabine D1-D14 of 3 week cycle

***Table I3 Cytotoxic chemotherapy regimens used in trials with bevacizumab in mCRC*:** IFL regimen used in Hurwitz 2004; FOLFIRI regimen used in Stathopoulos 2010 and Passardi 2015; FOLFOX4 and XELOX regimen used in Saltz 2008 and Giantonio 2007. FOLFOX6 or FOLFIRI used in Masi 2015

Aflibercept

Aflibercept is a recombinant fusion protein, containing the VEGF-binding domain of VEGFR-1 and VEGFR-2 fused to the Fc region of immunoglobulin G. It acts as a soluble decoy receptor and prevents binding of VEGF-A, VEGF-B and PlGF to their receptors to inhibit angiogenesis (Moroney et al 2009). Its use was evaluated in the phase III VELOUR study, in which aflibercept was combined with FOLFIRI in the second line treatment of metastatic colorectal cancer. Patients were randomised to FOLFIRI with aflibercept or placebo until disease progression. A small overall survival advantage was seen in the group

receiving aflibercept (13.5 versus 12.1 months, HR 0.82, 95% CI 0.71 to 0.94, p = 0.003) with a PFS improvement of 6.9 versus 4.7 months (HR 0.76, 95% CI 0.66 to 0.87, p = 0.00007) (Van Cutsem et al 2012).

Regorafenib

Regorefanib is an oral multikinase inhibitor of VEGFR1, VEGFR2, VEGFR3, TIE2, PDGFR and FGFR, KIT, RET, RAF1 and BRAF. The drug was most recently evaluated for single agent use after progression following all standard therapies. The phase III placebo controlled CORRECT trial found a statistically significant improvement in overall survival with the use of regorafanib (6·4 versus 5·0 months, HR 0.77, 95% CI 0.64-0.94, p=0.005) (Grothey et al 2013). Interestingly, the authors noted that main effect of regorafenib seemed to be disease stabilisation, rather than tumour shrinkage, illustrating the limitations of the traditional method of monitoring disease response using CT scanning and RECIST criteria with these targeted agents.

Ramucirumab

Ramucirumab is a human IgG-1 monoclonal antibody that targets the extracellular domain of VEGF receptor-2. The efficacy and safety of this drug was recently evaluated in a randomised phase III trial in combination with second-line FOLFIRI for metastatic CRC in patients with disease progression during or after first-line therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine. There was a small but statistically significant increase in median overall survival with the addition of ramucirumab (13·3 versus 11.7 months, HR 0·844 95% CI 0.73 to 0.97, p=0·022) (Tabernero et al 2015).

The addition of targeted therapies to conventional chemotherapy regimens in these settings has led to improvements in survival in sub-groups of patients, at the cost of further toxicities. It is crucial that robust biomarkers are sought to help classify patients according to these sub-groups and to maximise the chance of response whilst minimising exposure to significant toxicity.

Biomarkers in metastatic colorectal cancer

A biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.' The American Society of Clinical Oncology (ASCO) issued updated guidance on use of tumour markers in colorectal cancer in 2006 (Locker et al 2006) and the European Group on Tumour Markers issued guidance for the use of tumour markers in colorectal cancer in 2007 (Duffy et al 2007). They concluded that a lack of specificity and sensitivity precluded the use of all existing serum markers for the early detection of CRC but that faecal occult blood testing may be used to screen asymptomatic subjects over 50 years of age. Carcinoembryonic antigen (CEA) should be measured following resection of stage II or stage III CRC, if the patient is fit enough to consider further treatment in the case of disease recurrence.

Current biomarkers in metastatic colorectal cancer

A wide array of circulating markers has been investigated but only serum CEA has been validated for routine disease monitoring. It predicts for poor prognosis in advanced colorectal cancer patients (Park et al 1999) and is used to monitor response to therapy as well as monitor for disease relapse. Early detection of relapsed disease by CEA has been shown to lead to improvement in survival (Renehan et al 2002). However, CEA cannot predict response to conventional or targeted therapies.

Recent advances in biomarker discovery have led to significant changes in the treatment of metastatic colorectal cancer. Mutations in the K-RAS oncogene predict for resistance to EGFR-targeted therapies (Karapetis et al 2008). In this seminal study, patients with wild-type K-RAS colorectal cancer had a significant improvement in OS with single agent cetuximab (9.5 v 4.8 months) but this difference was not seen in patients in whom an exon 2 mutation was detected. Of note, the response rate to cetuximab was significantly higher in wild-type tumours than in mutated tumours (12.8 v 1.2%). These mutations were observed in 42% of the patients tested, representing a significant proportion of the patient population. K-RAS mutations are now routinely screened for prior to treatment with anti-EGFR therapies, such as cetuximab, illustrating the vital role of robust predictive biomarkers in improving patient outcomes and in minimising avoidable toxicities.

CT scanning is routinely used to monitor response to therapy in metastatic colorectal cancer, as in many solid cancers. The response assessment is based on the RECIST criteria, which was developed for use in monitoring disease treated with cytotoxic agents, rather than the targeted, cytostatic agents that are increasingly used today (Eisenhauer et al 2009). However, evidence is emerging that the RECIST criteria may not provide a suitable endpoint in studies using bevacizumab in combination with chemotherapy for the treatment of metastatic colorectal cancer. For example, analysis of the 2004 Hurwitz study revealed that CT based response rate did not predict for the magnitude of PFS or OS benefit from the addition of bevacizumab to the standard treatment (irinotecan and 5FU chemotherapy) (Grothey et al 2008). Similarly, in the 2008 Saltz study, the addition of bevacizumab to FOLFOX chemotherapy improved PFS without significantly improving response rates. A retrospective analysis of 82 patient data sets found that response by RECIST criteria was not associated with improved PFS or OS in metastatic colorectal cancer treated with bevacizumab and chemotherapy (PFS 28 months in partial responders versus 22 months in patients with stable or progressive disease $p = 0.45$) (Chun et al 2009) The raises the possibility that size criteria alone may not be a useful measure of response when treatment includes molecularly targeted agents. Alternatives to RECIST have been tested, including the Choi criteria, which define partial response as a decrease in size of at least 10% or a decrease in tumour attenuation of at least 15% on CT and progressive disease as an increase in tumour size of at least 10% without a decrease in tumour attenuation on CT. These criteria have been used in the treatment of GIST with imatanib (Choi et al 2007) and have shown to be of some clinical use in the treatment of metastatic renal cell cancer with sunitinib.

Circulating markers of angiogenesis

In view of the high rates of VEGF-A expression in colorectal cancer, the VEGF family has been widely evaluated as potential biomarkers. It has been reported that plasma VEGF concentrations are significantly higher in patients with advanced disease (Werther et al 2000) or who have higher metastatic disease burden (Davies et al 2000). A recent study aimed to ascertain whether preoperative serum VEGF levels correlated with clinical outcomes. They found that although VEGF concentrations significantly correlated with tumour size and CEA level, they did not predict overall survival (Kwon et al 2010). This contrasted with another study (Broll et al 2001) in which the serum concentration was of

prognostic significance and a further investigation that linked low concentrations of VEGF with better prognosis as well as improved response to chemotherapy (Hyodo et al 1998).

There are an increasing number of potential prognostic and predictive biomarkers reported in trials, all of which require further investigation if they are to be validated for clinical use. The following circulating angiogenesis-related proteins were investigated as part of the study entitled '*The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer'*. Multiplex ELISA technology was used to measure serum concentrations of vascular endothelial growth factors A, C and D; VEGF receptors 1 and 2; angiopoietin-2, Tie2, basic fibroblast growth factor, hepatocyte growth factor, placental growth factor, stromal-derived factor-1, interleukins 6 and 8, keratinocyte growth factor, E selectin and vascular cell adhesion molecule 1. *Table I4* summarises the rationale for inclusion of these proteins in the study. These proteins were of interest within the research group at the time of designing the studies, as they are associated with angiogenesis and, in particular, with angiogenesis in CRC.

CIRCULATING FACTOR	PREVIOUS STUDIES	REFERENCES
VEGF-A	High VEGF-A correlate with disease burden. Some evidence that low levels correlate with improved survival.	Davies 2000 Hyodo 1998 Broll 2001
VEGF-C	Angiogenesis associated protein	Tammela 2008
VEGF-D	Low tumour expression of VEGF-D predicts for response to chemotherapy plus bevacizumab	Weickhardt 2015
VEGFR-1	Pre-treatment serum VEGFR-1 is associated with primary tumour stage and adverse events after neo-adjuvant bevacizumab and chemo-radiation for rectal cancer	Duda 2010
VEGFR-2	Main mediator of VEGF-induced angiogenic signalling in cancer	Ferrara 2003
FGFb	Serum levels rise prior to progressive disease by RECIST criteria	Kopetz 2010
Interleukin 6	Serum IL6 correlates with outcome in neo-adjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer	Willett 2009
Interleukin 8	High pre-treatment serum IL8 associated with a shorter PFS	Kopetz 2010
Angiopoietin 1	Ang1 alters tumour growth by stabilizing blood vessels or by promoting angiogenesis in a pre-clinical CRC model	Satoh 2008
Angiopoietin 2	Low pre-treatment Ang2 is associated with improvement in PFS with bevacizumab therapy	Goede 2010
Tunica Internal Endothelial Kinase 2	Serum levels of Tie-2 receptor correlate with risk of development of metastatic disease	Chin 2003
Placental Growth Factor	High PlGF correlates with shorter survival	Wei 2005 Wei 2009
Platelet Derived Growth Factor	Serum PDGF-bb down-regulated in CRC patients compared to controls	Braicu 2013
Hepatocyte Growth Factor	Serum levels rise prior to progressive disease by RECIST criteria	Kopetz 2010
Keratinocyte Growth Factor	Over-expression of endogenous KGF genes in CRC cells increase VEGF-A production	Narita 2009
Stromal Cell-Derived Factor 1b	Serum levels rise prior to progressive disease by RECIST criteria	Kopetz 2010
Endothelial-Leucocyte Adhesion Molecule 1	Serum E-selectin elevated in mCRC patients compared with controls Pre-operative E-selectin correlates with survival in early CRC. Serum levels correlate with disease stage	Wittig 1996 Alexiou 2001
Vascular Cell Adhesion Molecule 1	Pre-operative VCAM-1 correlates with survival in early CRC. Serum levels correlate with disease stage	Alexiou 2001

Table I4: Circulating angiogenesis-related proteins evaluated as part of this research

ANGIOGENESIS IN EPITHELIAL OVARIAN CANCER

OVARIAN CANCER

Epidemiology

Over 6300 new cases of ovarian cancer were diagnosed in the UK in 2014 (Cancer Registration Statistics, England 2014). In early disease, complete surgical excision is often possible and the prognosis is favourable. Unfortunately, at least 65% of patients present with advanced disease (FIGO stage III or IV). In those presenting with stage III disease, five year survival is 19% and with stage IV only around 3% (CRUK: Ovarian cancer survival data 2002-2006). Despite investigations into various methods, no screening test has yet been found to be effective in improving survival, despite an improvement in early detection rates (Menon et al 2009; Jacobs et al 2016).

The aetiology of epithelial ovarian cancer is complex and several hormonal and reproductive factors have been postulated to be linked to risk. These include early menarche, late menopause, therapeutic use of hormone therapy, parity and infertility. Although most ovarian cancers are thought to be sporadic in aetiology, personal or family history of breast or ovarian cancer does confer a significant risk. Around 10% of ovarian cancer cases will be attributed to genetic factors including BRCA1 or BRCA2 mutations or HNPCC (Li and Karlan 2001).

Staging and surgical management of ovarian cancer

Staging of ovarian cancer is surgical, following the International Federation of Gynaecology and Obstetrics (FIGO) guidelines. Optimal surgical staging must include midline laparotomy, total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic omentectomy, biopsies of any peritoneal deposit, random biopsies of the pelvic and abdominal peritoneum and assessment of retroperitoneal lymph nodes. The proposed study will focus on patients with relapsed, platinum-sensitive epithelial ovarian cancer.

Cytotoxic management of advanced ovarian cancer

Surgical cytoreduction and cytotoxic chemotherapy form the basis of primary therapy for advanced ovarian cancer. Many patients will achieve complete clinical remission following primary therapy but, unfortunately, recurrence is common with many patients undergoing multiple lines of cytotoxic therapy until acquired drug resistance limits therapeutic options.

As the majority of patients with ovarian cancer present with advanced disease, it is vital that new therapeutic strategies are explored to overcome this resistance to chemotherapy and to prolong remission duration.

Platinum therapy forms the basis of the cytotoxic management of ovarian cancer. This was originally based on the GOG 47 trial, published in 1986, which showed significantly prolonged OS in cancer patients with advanced, measurable disease in whom cisplatin was added to the control arm of doxorubicin/cyclophosphamide (19.7 versus 15.7 months) (Omura et al 1986). Carboplatin was evaluated as an alternative to cisplatin as it is more tolerable, particularly with regards to nephro- and neurotoxicity. A Cochrane review of all available trials showed no difference in overall survival between carboplatin and cisplatin (HR of 1.02, 95% CI 0.93-1.12) (Stewart et al 1999).

Standard first line treatment in fit patients (EGOG performance status 0 to 1) in the UK is now a combination of carboplatin and paclitaxel, given 3-weekly. This is on the basis of large trials such as the GOG 111 trial (Piccart et al 2000) and a similar European–Canadian Intergroup study (Muggia et al 2000) which both showed significantly prolonged OS with cisplatin/paclitaxel compared with cisplatin/cyclophosphamide. This finding has not been replicated in all subsequent studies. For example, the ICON3 study (n = 2074) randomised to three arms: single agent carboplatin, carboplatin/paclitaxel or cisplatin/doxorubicin/cyclophosphamide and did not show a significant outcome difference between the arms (ICON group 2002). A meta-analysis of four large randomised trials suggested a benefit with the addition of paclitaxel to platinum therapy but noted significant heterogeneity in the results as well as increased toxicity (Sandercock et al 2002). This raises questions as to who is most likely to benefit from paclitaxel treatment and whether future markers could identify these sub-groups.

The addition of a third chemotherapeutic agent, such as liposomal doxorubicin or topotecan, to carboplatin and paclitaxel did not alter outcomes in the large GOG 182-ICON5 trial (n= 4000) but did lead to poor tolerability, particularly due to haematological toxicities (Bookman et al 2009). This has increased interest in the use of combinations including targeted therapies as an alternative to triplet regimens of conventional chemotherapeutic agents. Alternative strategies for drug administration are also being explored currently, with encouraging results with the use of ‘dose-dense’ weekly therapies

and intraperitoneal chemotherapies. It is clear that, whilst improvements are being made, continued work to understand the biology of ovarian cancer is vital to reduce cytotoxic resistance and to improve survival.

Angiogenesis in ovarian cancer

Angiogenesis has a key role in the physiological process of the female reproductive cycle and is necessary for follicular development. Intrafollicular levels of VEGF proteins increase during the initial phase of the ovulatory cycle and peak immediately prior to the luteal phase. Increasing levels of the angiogenic inhibitor angiopoietin-2 are associated with vessel regression and follicular degeneration towards the end of the luteal phase (Ramakrishnan et al 2005).

Aside from the physiological response of the ovary to angiogenic regulation, it is now clear that angiogenesis is crucially important in the pathophysiology of ovarian cancer. High levels of VEGF have been found in ovarian cancer cells and VEGF over-expression appears to confer a poor prognosis in patients with primary ovarian cancer (Duncan et al 2008). Conversely, decreased VEGF expression correlates with reduced angiogenesis, lower ascitic fluid volume and improved survival in mice (Huang et al 2000). These findings have led to a keen interest in exploiting the VEGF pathways in the treatment of ovarian cancer with the aim of inhibition of angiogenesis and, in turn, regression of ascites and tumour bulk.

VEGF inhibition in advanced epithelial ovarian cancer

Bevacizumab

Bevacizumab was the first VEGF-targeting agent to be incorporated into large phase III trials in ovarian cancer, following two phase II studies showing significant single agent efficacy in patients with recurrent disease. The first of these phase II studies involved the administration of single agent bevacizumab 15mg/kg every 3 weeks intravenously until disease progression in 62 patients with measurable, relapsed epithelial ovarian cancer. It reported a 21% radiological response rate and a median progression free survival of 4.7 months (Burger et al 2007). The second study enrolled 44 patients with heavily pre-treated, platinum resistant disease. This group used the same dosing regimen of bevacizumab and found a radiological response rate of 15.9% and median progression free survival of 4.4

months. The study closed early due to excessive toxicity, with a gastrointestinal perforation rate of 11.4% and an arterial thromboembolic event rate of 6.8% (Cannistra et al 2007). Nevertheless these data were particularly interesting as single agent responses were seen in many platinum resistant tumours, in which treatment options are limited.

Two large randomised phase III trials in the first line setting have recently been reported (*figure 11*). The GOG-218 study recruited 1873 patients with epithelial ovarian cancer, who had stage III disease with microscopic disease, residual tumour or stage IV disease, following primary debulking surgery (Burger et al 2011). Patients were randomised into three arms: I- standard carboplatin and paclitaxel chemotherapy alone; II- chemotherapy with concurrent bevacizumab 15mg/kg or III- chemotherapy with both concurrent and maintenance bevacizumab for up to 15 months. The primary end-point of PFS showed a statistically significant improvement in PFS in arm III compared to arm I (median PFS arm III 14.1 v arm I 10.3 months, HR 0.72, 95% 0.63 to 0.82, p <0.0001). Outcome comparisons with arm II showed no significant differences in PFS, suggesting that the principal benefit is derived from maintenance bevacizumab rather than concurrent treatment. There was no significant difference in overall OS, although there was an improvement in OS in patients with ascites (unplanned sub-group analysis, HR 0.82, 95% CI 0.70 to 0.96, p = 0.014) (Ferriss et al 2015). The OS results may be confounded by the use of post progression anti-angiogenic agents in the control arm. Notably, the rates of gastrointestinal toxicities of grade 2 or higher were reported at around 2.8%; much lower than in the phase II studies. This may reflect the first-line nature of this study in contrast to the heavily pre-treated phase II cohort of patients.

The ICON7 trial also evaluated bevacizumab in combination with first line cytotoxic chemotherapy but at the lower dose of 7.5mg/kg every 3 weeks (Perren et al 2011). ICON7 had two treatment arms: arm I had standard chemotherapy and arm II had the addition of bevacizumab during chemotherapy and as maintenance, totalling 18 cycles of bevacizumab. It included patients with early disease and, unlike GOG-218, did not consider isolated CA125 rises as a marker for progression. The ICON group also reported a modest but significantly improved median PFS (arm I 17.3 months v arm II 19.0 months p = 0.0041) but notably the survival curves cross at around 21 months. In long-term follow up, a pre-planned sub-group analysis of those patients considered to be at high risk of relapse (stage III/IV with 1cm or more of residual disease) showed a significant difference

in overall survival with the addition of bevacizumab to chemotherapy (OS 34·5 months versus 39·3 months with bevacizumab; log-rank p=0·03) (Oza et al 2015). This may be expected as angiogenesis is not required by micrometastases as it is in bulky disease.

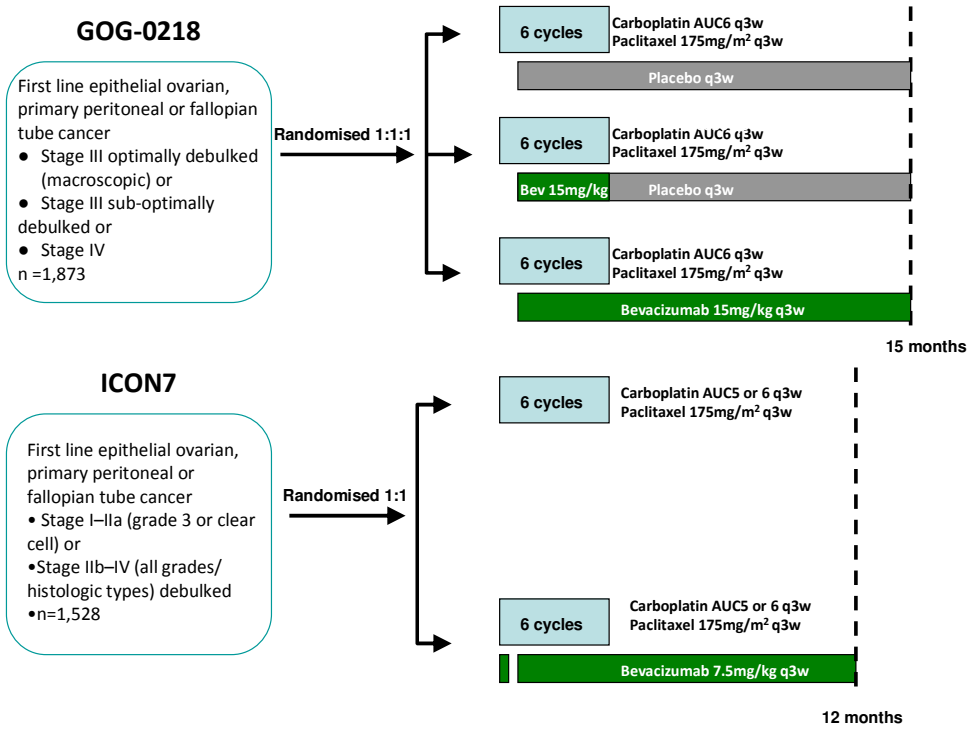

Figure I1. Study schema for GOG-0218 and ICON7 studies. Note that the both the inclusion criteria and the bevacizumab dose differ between the two studies.

Bevacizumab has also been used in combination with cytotoxic chemotherapy in the second line treatment of ovarian cancer. The phase III OCEANS trial randomised patients with platinum-sensitive ovarian cancer to receive carboplatin and gemcitabine with or without bevacizumab 15mg/kg every 3 weeks. This showed a significant improvement in PFS (12 versus 8 months). However, OS was not significantly different between two arms but toxicity was significantly higher in the bevacizumab arm (in particular hypertension and proteinuria) (Aghajanian C et al 2012). Bevacizumab has also been shown to improve PFS, but not OS, in combination with cytotoxic chemotherapy in platinum-resistant ovarian cancer. Patients were treated with pegylated liposomal doxorubicin, weekly

paclitaxel, or topotecan with or without bevacizumab at a dose of 10 mg/kg every 2 weeks or 15 mg/kg every 3 weeks, leading to an improvement in PFS from 3.4 months with chemotherapy alone versus 6.7 months with bevacizumab (Poveda et al 2015).

The data from these studies have raised questions as to the optimum duration of bevacizumab therapy in patients with ovarian cancer but also highlight the importance of identifying the subgroups most likely to derive benefit from treatment. To answer these important questions using a traditional model of another large randomised control trial is both time consuming and expensive. Validated biomarkers that are predictive for early response could help guide decisions in this setting and reduce the toxicity and expense incurred by treating patients who unlikely to benefit from this therapy.

Aflibercept

Aflibercept has shown some single agent efficacy in a phase II study of 215 patients with advanced epithelial ovarian cancer, following three or four lines of previous therapy (Gotlieb et al 2012). This study reported a radiological response rate of 7.3 % and a median progression free survival of 13.3 weeks using a dose of 4mg/kg/2 weeks. Interestingly, of the forty patients with evaluable ascites at baseline, 77.5% had stabilisation or reduction of their ascites. However, a further randomised phase II trial in patients with recurrent, platinum-resistant ovarian, peritoneal, or fallopian tube cancer who developed disease progression after receiving topotecan and/or pegylated liposomal doxorubicin did not meet its primary endpoint for response (clinical benefit rate (ORR plus stable disease >6 months) was 12.3% and 11% in the 2-mg/kg and 4-mg/kg cohorts, respectively (Tew et al 2014).

VEGF receptor tyrosine kinase inhibitors

Cediranib

Cediranib is an oral tyrosine kinase inhibitor of VEGFR-1, -2 and -3 as well as c-kit. It has single agent activity in recurrent epithelial ovarian cancer (Drevs et al 2007). A phase II study enrolled 47 patients with recurrent platinum resistant (65%) or sensitive (35%) disease and used single agent, continuous daily cediranib 45mg initially, dropping to 30mg after significant toxicities were observed in the first 11 patients. The primary end point was clinical benefit rate, defined as complete response, partial response or stable disease for greater than 16 weeks or CA125 non-progression for greater than 16 weeks. By this

definition 30% of patients derived a clinical benefit. Twenty-three percent of patients were removed from the study because of side effects. Notable grade 3 toxicities included hypertension (46%), fatigue (24%) and diarrhoea (13%). Grade 4 toxicities included CNS haemorrhage, altered lipid profile and elevated creatinine (n=1 for each). The median progression free survival was 5.2 months (Matulonis et al 2009).

On the basis of these data, the role of cediranib in combination with standard chemotherapy in platinum sensitive disease was explored as part of the recently reported ICON6 trial (Ledermann et al 2016). This trial sought to define the benefit incurred by adding cediranib to cytotoxic chemotherapy and then as maintenance therapy. It is important to note that the dose of cediranib was reduced during the trial to 20mg/day to improve the safety profile of the combination regimen. The trial was a randomised, three-arm, double-blind, placebo-controlled phase 3 trial, in which patients with relapsed platinum-sensitive ovarian cancer received up to six cycles of platinum-based chemotherapy (once every 3 weeks) then entered a maintenance phase. Participants were randomly allocated (2:3:3) to one of three arms:

Arm A: Chemotherapy plus placebo then placebo maintenance

Arm B: Chemotherapy plus cediranib 20mg od then placebo maintenance

Arm C: Chemotherapy plus cediranib 20mg od then cediranib 20mg od maintenance until disease progression or until the accumulation of excessive toxic effects.

The primary efficacy endpoint was progression-free survival between arms A and C. A total of 456 patients were randomised to receive chemotherapy with or without cediranib. Median progression-free survival was significantly prolonged by cediranib with a median PFS of 8.7 months in arm A versus 11.0 months in arm C (hazard ratio 0.56, 0.44–0.72, $p<0.0001$). Median overall survival was 21.0 months in arm A versus 26.3 months in arm C (HR 0.77, 95% CI 0.55–1.07, $p=0.11$). This OS data is considered immature at present and will require further follow up. Diarrhoea, neutropenia, hypertension, and voice changes were significantly more common during chemotherapy with cediranib, and diarrhoea, hypothyroidism and voice changes were more common during cediranib maintenance. The authors concluded that cediranib gives a meaningful improvement in PFS, when given with chemotherapy and as maintenance therapy.

The ICON6 trial forms the basis of a paper within this thesis, *'Evaluation of dynamic contrast-enhanced MRI for monitoring response to cediranib with cytotoxic therapy in*

platinum-sensitive ovarian cancer,' which investigates the potential role of dynamic contrast-enhanced (DCE-) MRI and diffusion weighted (DW-) MRI as biomarkers in patients treated with chemotherapy and cediranib. Functional MRI techniques are increasingly used within clinical trials of novel agents as a non-invasive means of characterising the tumour microenvironment and are described in more detail below.

Several other tyrosine kinase inhibitors have also been evaluated for the treatment of epithelial ovarian cancer (see *Table I5*). To date, none of these agents have gained a licence for use in this setting.

Agent	Targets	Current trials	Key findings to date	References
Nintedanib BIBF 1120	VEGFR-1, 2 and -3, PDGFR, FGFR-1 -2 and -3 and flt-3	Phase III completed AGO-OVAR 12	Carboplatin and paclitaxel +/- nintedanib with chemotherapy and as maintenance therapy following debulking of EOC. PFS 17·2 months vs 16·6 months, p=0·024 nintedanib v placebo	Du Bois 2010 Lederman n 2011 Du Bois 2016
Pazopanib GW786034	VEGFR1-3, PDGFR, FGFR-1 and -3 c-kit	Phase III completed AGO-OVAR 16	Maintenance therapy following first line chemotherapy in advanced EOC PFS 17.9 v 12.3 months in pazopanib v placebo	Freidlander 2010 Du Bois 2013
Sunitinib SU11248	VEGFR, EGFR, PDGFR, c-kit	Phase II completed	ORR in a non-randomised phase II trial was 8.3%	Biagi 2011 Bauman 2012 Campos 2013
Trebananib AMG386	Ang1 and Ang2	Phase III completed	Advanced EOC (PFI less than 12 months) with weekly paclitaxel. PFS significantly improved (7.2 v 5.4 months). OS not improved (19.3 v 18.3 months)	Monk 2014 Amgen Press Release 2014

Table I5: Selected tyrosine kinase inhibitors currently under evaluation for use in epithelial ovarian cancer. VEGF: Vascular endothelial growth factor, PDGFR: Platelet derived growth factor receptor, FGFR: Fibroblast growth factor receptor, EGFR: Epithelial growth factor, Ang: angiopoietin

BIOMARKERS IN OVARIAN CANCER

Current biomarkers in advanced ovarian cancer

Serum CA125 antigen is the only circulating biomarker that is commonly used to guide the management of epithelial cancer in the UK. It is used in combination with pelvic ultrasound and menopausal status to form the 'risk of malignancy index' for the diagnosis of ovarian cancer (Tingulstad et al 1996). CA125 measurements alone are less accurate in detecting early disease, where only around 50% of patients will have an abnormally high level (Jacobs and Best 1989). Its role in screening has been hampered by this and by its low specificity, with around 6% of women with high CA125 levels having no malignancy at all (Einhorn et al 1992). The role of CA125 in combination with annual transvaginal ultrasound screening (multimodal screening, MMS) has been recently investigated as part of the randomised UKCTOCS trial, in which 202 638 women were randomised between MMS, ultrasound alone or no screening. The mortality reduction was not significant in the primary analysis, but the authors noted a significant mortality reduction with MMS when prevalent cases were excluded in a pre-planned analysis. They concluded that further follow-up is needed before conclusions can be reached on the efficacy of MMS (Jacobs et al 2016).

CA125 is validated for the monitoring of advanced disease, with over 80% of such patients showing high serum CA125 levels. Serum CA125 levels are of prognostic significance, with high Ca125 levels prior to cycle three of chemotherapy being associated with a poor prognosis in 57% of patients (Fayers et al 1993). However, in the remaining 43% of patients, a decision to alter treatment at cycle three may well have been unnecessary, and indeed incorrect. The OVO5 trial showed no evidence of a survival benefit with early treatment of relapse on the basis of a raised CA125 concentration alone in patients who attain a complete response after first-line treatment (Rustin et al 2010). There has been subsequent debate surrounding the benefit of using CA125 levels alone in the routine follow up of advanced ovarian cancer. Combined CA125 and CT-based RECIST criteria can be combined to monitor ovarian cancer response in the trial setting, according to Gynaecological Cancer Intergroup (GCIG) guidelines (Rustin et al 2011). CA 125 response is defined as at least a 50% reduction in CA 125 levels from a pre-treatment sample. The response must be confirmed and maintained for at least 28 days and the pre-

treatment result must be at least twice the upper limit of the reference range within 2 weeks of starting treatment.

Markers of angiogenesis in ovarian cancer

The significant toxicities associated with the use of agents targeting the angiogenic pathways illustrate the importance of selecting patients who are most likely to gain benefit; hence the need for prognostic and predictive biomarkers. Microvessel density (MVD) analysis of ovarian tumours by IHC has been shown to provide useful prognostic information, with high MVD correlating with low PFS and OS (Merritt and Sood 2007). However, the clinical utility of this technique for disease response monitoring is clearly limited by the invasive nature of multiple tumour biopsies.

A phase II trial of patients with recurrent ovarian cancer treated with bevacizumab evaluated the role of plasma VEGF levels as a prognostic marker. Pre-treatment VEGF concentrations did not correlate with patient outcome and although the plasma concentrations declined with bevacizumab treatment, this decline was not associated with improved response rates (Han et al 2010). These findings were explored further as part of the phase III GOG-218 study, in which the plasma of 582 patients was analysed for VEGF and VEGFR-2. Plasma was drawn following surgery for advanced ovarian cancer, prior to the initiation of chemotherapy plus or minus bevacizumab. This study did not reveal an association between these angiogenic markers and patient outcome (Birrer et al 2012). Circulating angiogenesis-related proteins were also analysed as part of the ICON7 study of chemotherapy plus bevacizumab in ovarian cancer. This translational study showed the combined values of angiopoietin 1 and tunica internal endothelial cell kinase 2 are predictive biomarkers for improved PFS (Backen et al 2014).

Despite work to investigate novel circulating biomarkers in ovarian cancer, their role remains limited at present. It is therefore important that other non-invasive methods, such as functional imaging, are explored in parallel with the on-going serological based investigations.

APOPTOSIS

Apoptosis is an active process, characterised by cell shrinkage, plasma membrane blebbing, chromatin condensation and fragmentation, leading to cell death. All remaining products are removed from the microenvironment by phagocytosis. This closely regulated process contrasts sharply with necrotic cell death, in which cellular contents are released into surrounding tissues and lead to an inflammatory response (*Table I6*). Evasion of apoptosis, or programmed cell death, is a hallmark of cancer (Hanahan and Weinberg 2011).

APOPTOSIS	**NECROSIS**
Membrane blebbing with no loss of integrity; chromatin condensation and fragmentation and formation of apoptotic bodies.	Loss of cell membrane integrity with total cell lysis and disintegration of organelles.
Energy dependent	No energy requirement
Non-random DNA fragmentation	Random DNA digestion.
Caspase dependent	Not regulated by caspases
Induced by physiological stimuli (e.g. lack of growth Factors or changes in hormonal environment)	Induced by non-physiological stimuli (e.g. hypothermia, hypoxia or ischaemia)
No inflammatory response	Significant inflammatory response

Table I6: Key differentiating features of modes of cell death. Apoptosis is a closely regulated process, leading to cell death and removal of cell products by phagocytosis.

Regulation of apoptosis

Cells may be induced to undergo apoptosis by extracellular 'death factor' signals, via the extrinsic pathway, or by intrinsic physical or chemical stresses. These pathways rely on the regulatory effects of cysteine–rich aspartate proteases (caspases) that cleave intracellular proteins at aspartate residues. Caspases can be divided into initiators, which activate other caspases, and effectors, which cleave substrates during apoptosis. A caspase cascade is established in which one caspase initiates the next, leading to amplification of the original signal (see *figure I2*). Of note, both the intrinsic and extrinsic pathways converge and rely on caspases 3, 6 and 7 to initiate apoptosis. This convergence is exploited in the 'death switch' project, in which tumour cells are induced to undergo synchronous apoptosis by activation of caspase 3.

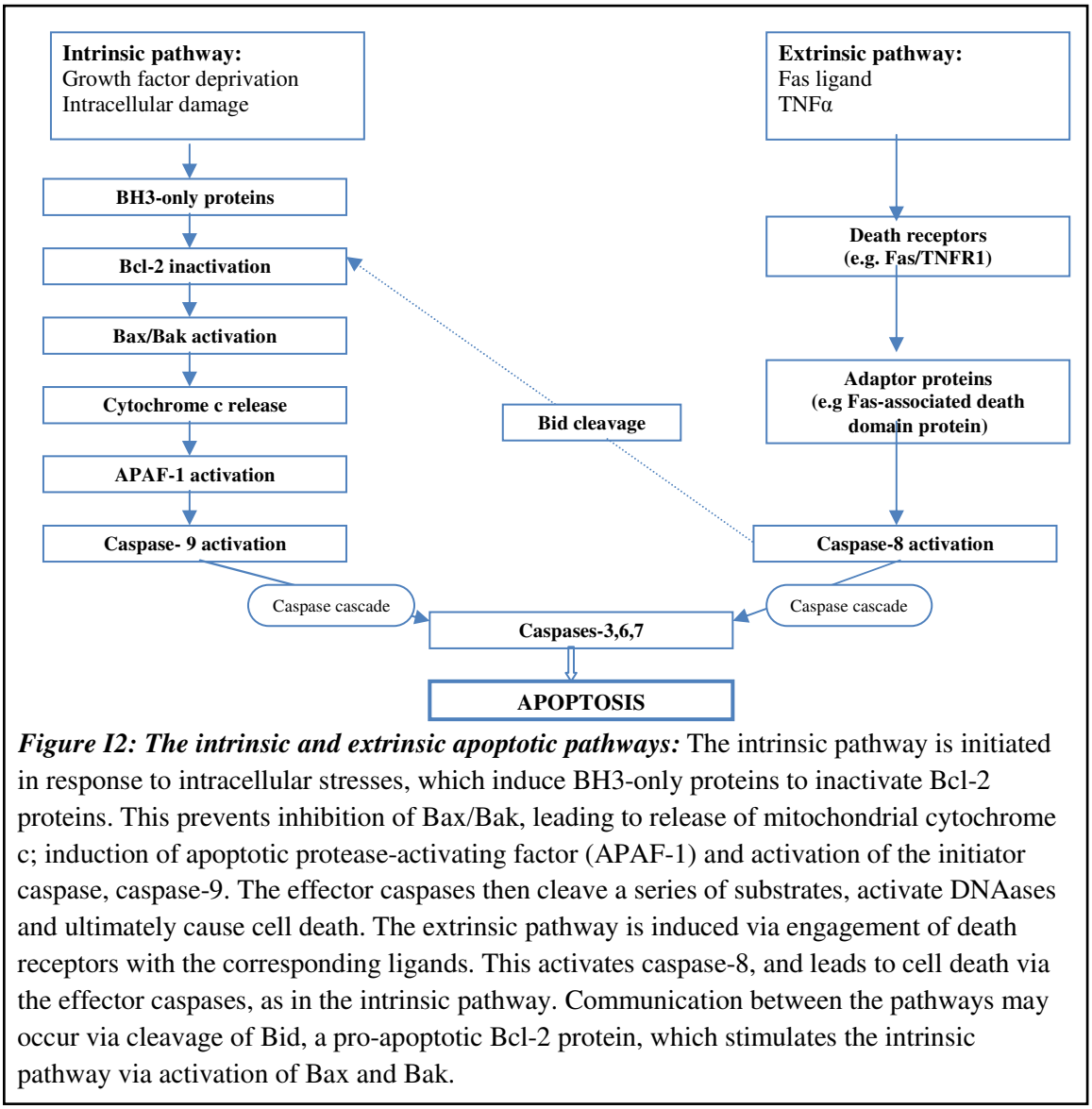

Figure I2: The intrinsic and extrinsic apoptotic pathways: The intrinsic pathway is initiated in response to intracellular stresses, which induce BH3-only proteins to inactivate Bcl-2 proteins. This prevents inhibition of Bax/Bak, leading to release of mitochondrial cytochrome c; induction of apoptotic protease-activating factor (APAF-1) and activation of the initiator caspase, caspase-9. The effector caspases then cleave a series of substrates, activate DNAases and ultimately cause cell death. The extrinsic pathway is induced via engagement of death receptors with the corresponding ligands. This activates caspase-8, and leads to cell death via the effector caspases, as in the intrinsic pathway. Communication between the pathways may occur via cleavage of Bid, a pro-apoptotic Bcl-2 protein, which stimulates the intrinsic pathway via activation of Bax and Bak.

Apoptosis and chemotherapy

Cytotoxic chemotherapeutic agents are able to induce apoptosis in cancer cells. Chemotherapies induce DNA damage, triggering the intrinsic pathway and may also induce TNF production, leading to extrinsic pathway activation. Cells that are incapable of apoptosis are therefore resistant to standard chemotherapy regimens. Examples of mutations which lead to defective apoptotic pathways include p53 mutations and loss of Bax, a pro-apoptotic protein. Such mutations are associated with resistance to 5-fluoruracil in colorectal cancer cells (Zhang et al 2000).

Targeting apoptosis in metastatic colorectal cancer

Intact apoptotic regulation is crucial for mucosal development and homeostasis in the colon (Koehler et al 2014). Defective apoptotic signalling is implicated in the development of colorectal cancer, as well as in inflammatory bowel diseases (Levin et al 2016).

The pro-apoptotic BH3 family members that have shown differential expression between CRC tumours and normal mucosa include Bad, Bid and PUMA. Higher protein expression of Bad and Bid have been shown to correlate with longer overall survival in patients with stage II and III CRC treated with 5FU chemotherapy (Sinicrope 2008). PUMA has not been shown to be a useful biomarker in CRC. Bax and Bak trigger the release of cytochrome *c*, leading to caspase activation and apoptosis (Griffiths et al 1999). Bax expression is associated with superior survival in stage III (Nehls et al 2007) and in metastatic colorectal cancers (Sturm et al 1999). Following release from the mitochondria, cytochrome *c* complexes form the apoptosome with apoptotic peptidase activating factor-1 (APAF-1), which activates pro-caspase 9. Loss of APAF-1 expression at the protein level correlates with shorter overall survival (Zholbec et al 2007). The binding of TRAIL to its receptors DR4 and DR5 activates the extrinsic apoptotic pathway. Studies examining the levels of the TRAIL receptors DR4 and DR5 show higher expression of both receptors in colonic tumours compared to normal tissue (Koornstra et al 2003). Over expression of DR4, but not TRAIL or DR5 confers poor disease free survival in patients with Dukes C CRC (van Geelan et al 2006).

In normal colonic mucosa, the anti-apoptotic protein Bcl-2 is expressed exclusively in the base of colonic crypts where there are low levels of apoptosis (Watson 2004). This pattern is lost in the progression to colorectal cancer (Sinicrope et al 1995). In addition, over

expression of Bcl-2 has also been associated with resistance to 5FU, which forms the basis of treatment of metastatic colorectal cancer (Violette et al 2002). Bcl-2 has been studied as a potential prognostic biomarker in colorectal cancer with several studies showing an association between Bcl-2 protein over expression and prolonged survival in early stage colorectal cancers (Biden et al 1999; Krajewska et al 2005). This is perhaps surprising, given the anti-apoptotic effect of Bcl-2. However, other studies have shown either no correlation between Bcl-2 expression and survival (Garrity et al 2004) or that over expression of Bcl-2 correlates with shorter survival (Bhatavdekar et al 1997) and so the clinical utility of this marker remains unclear. Over expression of Survivin, a member of the IAP family inhibits apoptosis and several studies have shown poor survival rates in colorectal cancer with over expression of Survivin mRNA (Sarela et al 2000) and protein (Kawasaki et al 2008).

As tumour cells are typically resistant to death by apoptosis, re-initiation of cell death pathways is an important goal of anti-cancer drug development. Research is ongoing into strategies to activate pro-apoptotic signals and to overcome the mutations that cause resistance to pro-apoptotic drugs (Letai 2008) (*Table I7*).

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DRUG	TARGET	STUDY PHASE	CONCLUSIONS	REFERENCE
BV6 SMAC mimetic	IAP	Preclinical in vivo with radiation in 3D grown CRC cells	Induces apoptosis	Hehlgans 2015
Conatumumab	DR5	Phase Ib/II with FOLFOX6 and bevacizumab in mCRC	No improved efficacy with addition of trial drug	Fuchs 2013
Oblimersen	Bcl-2	Phase I with irinotecan in mCRC	Acceptable toxicities.	Mita 2006
Survivin peptide vaccine	Survivin	Phase I single agent in mCRC	Acceptable toxicities	Tsuruma 2004
Tigatuzumab	DR5	Phase I single agent in solid tumours	Acceptable toxicities.	Forero-Torres 2010

Table I7: An overview of recent clinical trials using drugs targeting the apoptotic pathway in colorectal cancer. IAP: inhibitor of apoptosis proteins (include Survivin, XIAP, cIAP1 and cIAP2). DR5: death receptor 5, activated by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).

Targeting apoptotic pathways in ovarian cancer

Platinum-based therapy forms the backbone for chemotherapy regimens in advanced ovarian cancer, with acquired resistance to platinum chemotherapy eventually leading to disease progression. Cisplatin up-regulates the pro-apoptotic factors p53, Fas, and Bax in a number of cell types (Gatti et al 1999). However, cisplatin also down-regulates anti-apoptotic proteins such as XIAP and Akt and chemo-resistance is likely to be a result of a loss in this regulatory balance (Li et al 2001). The function of the PI3K/AKT survival pathway is often altered in human ovarian cancer and high levels of PI3K and AKTs have

been linked to resistance to cisplatin (Cheung et al 2002) and to paclitaxel (Hu et al 2002). Bcl-2 and Bcl-XL over expression has been found in cisplatin-resistant ovarian cancer cell lines on both mRNA and protein levels. Therefore, it appears that chemo-resistance may also be associated with enhanced expression of these anti-apoptotic genes (Yang et al 2004). In the clinical setting, low Bcl-2 and high p53 expression, are significantly correlated with shorter survival (de la Torre et al 2007; Herod et al 1996).

Recent efforts have attempted to exploit these aberrations in apoptotic regulation with the use of drugs designed to reduced chemo-resistance. Examples include the Bcl-2/Bcl-XL family inhibitor ABT-737, which sensitizes ovarian cancer cells to carboplatin (Witham et al 2007) and oridonin, which reverses cisplatin drug resistance in ovarian cancer cells (Ma et al 2016). Lovastatin, has been found to induce apoptosis in ovarian cancer cells and synergizes with doxorubin *in vitro* (Martirosyan et al 2010). A phase II study of interaction of lovastatin and paclitaxel for patients with refractory or relapsed ovarian cancer is ongoing (NCT00585052, not yet reported).

Circulating markers of cell death

Serum was analysed for circulating cytokeratin 18 (CK18) levels in all three of the projects described in this thesis, as a measure of tumour cell death. The M30 Apoptosense and M65 ELISAs (*Peviva, Sweden*) detect CK18 fragments. The M30 assay selectively detects caspase cleaved CK18 (cCK18), released when apoptosis occurs, whereas the M65 assay detects total CK18 (tCK18), reflecting total cell death in the form of apoptosis and necrosis (Cummings et al 2006). These assays have previously been validated within the laboratory (Greystoke et al 2008).

The M30 and M65 assays have been previously evaluated in a pre-clinical colorectal cancer model, where cCK18 levels increased with anti-tumour treatment, correlating with an increase in apoptotic staining on IHC. Circulating tCK18 levels positively correlated with tumour burden suggesting that these markers may be useful as pharmacodynamic biomarkers of apoptosis and as biomarkers of therapeutic response (Cummings et al 2008). These assays were further evaluated in 74 patients with metastatic colorectal cancer (CRC) in a study in which the circulating levels of cCK18 and tCK18 were compared to those seen in 53 healthy volunteers (Greystoke et al 2012). Median cCK18 and tCK18 concentrations were significantly higher in those with a diagnosis of CRC compared with

controls (p = 0.0001 for both assays). High baseline tCK18 levels prior to first line chemotherapy for mCRC were associated with lower overall survival (p = 0.0017) and patients with progressive disease on therapy had elevated baseline serum tCK18. Pharmacodynamic changes were seen in these patients, with CK18 levels falling in response to treatment. This is in keeping with a study carried out in patients with resectable CRC, in whom resection of the tumour resulted in a significant decrease in tCK18 and cCK18 (Koelink et al 2009). This study also reported a significant decrease in disease-free survival in patients with high levels of serum CK18.

Serum CK18 levels are studied as part of the current clinical project '*The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer'* and in the preclinical setting ('*ADC biomarkers detect caspase induced apoptosis in HT29 colorectal xenografts'*). As the apparent diffusion coefficient derived from diffusion-weighted MRI reflects tumour apoptosis, one of the aims of these studies was to evaluate any relationship between serum CK18 levels and ADC.

CIRCULATING TUMOUR CELLS

In addition to soluble markers, we have investigated the relationship between circulating tumour cells (CTCs) and several MRI parameters. CTCs are present in the blood of cancer patients but are rare in healthy volunteers (Allard et al 2004). A 2008 study found that patients with metastatic CRC with more than three CTCs per 7.5ml of blood before treatment had a significantly worse PFS and OS than those with less than three cells (PFS 4.5 v 7.9 months and OS 9.4 v 18.5 months). Reductions in CTC number from over three to fewer than three cells per 7.5mls of blood after 3-5 weeks of treatment were associated with a significant improvement in PFS and OS. The study concluded that CTC number can be used as a prognostic marker in metastatic CRC and validated CellSearch technology in metastatic CRC (Cohen et al 2008). This technology has subsequently been approved for use by the Food and Drugs Administration.

This validated CTC technology was combined with DCE-MRI markers by drawing blood at the same time-points as taking MRI images in '*The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer'* study. This tested the hypothesis that tumour microvasculature changes seen on DCE-MRI correspond to changes in the number of CTCs released into the circulation in metastatic colorectal cancer. There are currently very limited data available surrounding any potential correlation between CTCs and MRI parameters. Interestingly, one small preclinical study did show that high K^{trans} was strongly correlated with metastatic potential of cells and that K^{trans} changes preceded an increase in CTCs in a non-small cell lung cancer model (Chou et al 2013).

DYNAMIC CONTRAST-ENHANCED MAGNETIC RESONANCE IMAGING

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a non-invasive, quantitative method of investigating the structure and function of tumour vasculature by tracking the pharmacokinetics of a paramagnetic contrast agent as it passes through the tumour vessels. DCE-MRI does not involve ionising radiation and can be performed on standard specification 1.5 Tesla clinical systems, making it an attractive technique for use in clinical practice.

The DCE-MRI imaging data are acquired in three stages; the first being used to localise the tumour and the surrounding tissues. Following this, sequences are acquired to allow calculation of baseline tissue T_1-values. The final stage is to administer contrast and record the dynamic sequence data, using scans every few seconds over around 5- 10 minutes. The contrast agent, a gadolinium chelate, is administered as a bolus injection via a large peripheral vein. The administration dose and rate are standardised across the patient group, using an automated pressure injection system, to minimise alterations in flow rate. The contrast agent enters the tumour arterioles, passes through the capillary bed and then drains via tumour veins. The paramagnetic nature of the gadolinium ions cause them to interact with surrounding hydrogen nuclei, shortening the T1-relaxation time in nearby tissues and leading to a measurable increase in signal intensity on T1-weighted images. The degree of this enhancement varies within each voxel and is dependent on several factors including tissue perfusion, contrast agent concentration, blood flow rate, the arterial input function (AIF, the concentration-time course of contrast agent in the artery supplying the tumour vasculature), vascular surface area and permeability, extravascular extracellular space (v_e) and the native T_1-relaxation times of each tissue. The parameters generated from DCE-MRI imaging can therefore be used to characterise changes in tumour blood flow, vascular permeability and extracellular extravascular and vascular volumes. *Figure I3* represents a compartmental model of the tumour vasculature and illustrates the movement of the contrast media following injection.

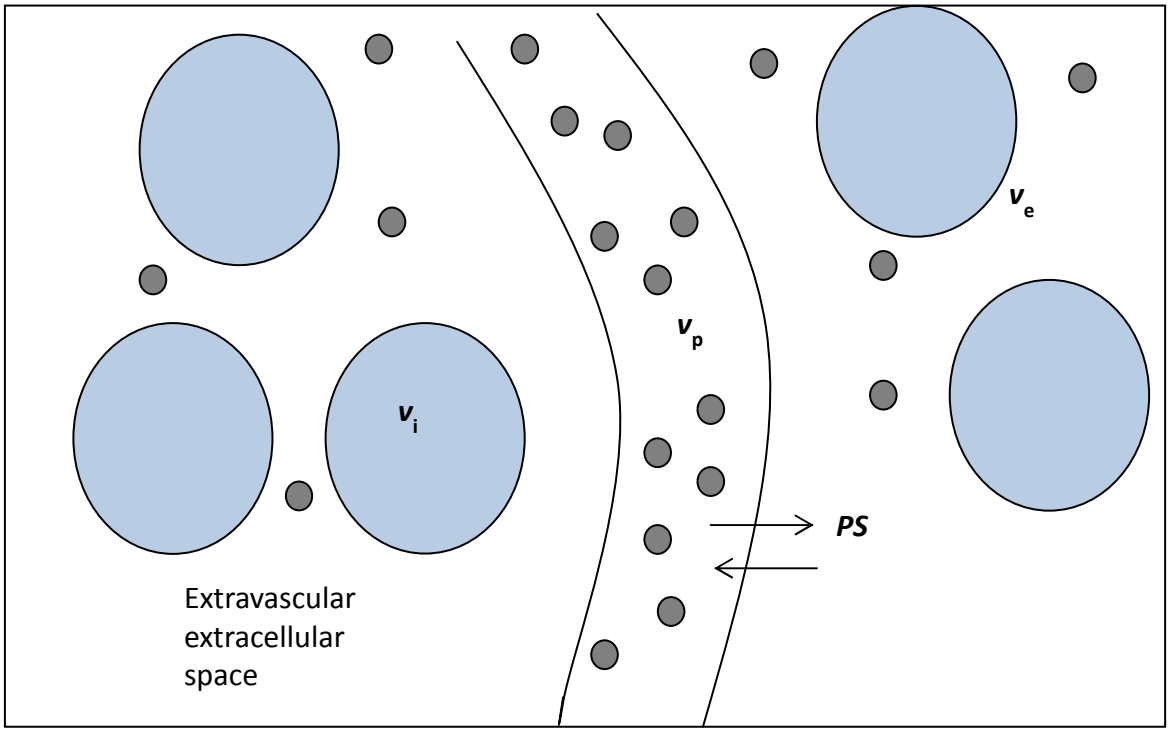

***Figure I3: Contrast agent movement in DCE-MRI*:** Contrast media molecules (represented as grey dots) enter the tumour vasculature and are distributed into two compartments – the blood plasma (measured by the parameter v_p) and the extravascular extracellular space (measured by v_e). The rate of contrast agent transfer between these spaces is represented by the calculated K^{trans} and is dependent on the rate of blood flow; the concentration difference of contrast agent between space v_p and space v_e; the vascular permeability and surface area of the tumour vessel. v_i represents in the intracellular space. PS is the endothelial permeability-surface product, which describes the permeability of the vessel.

Various parameters can be measured or derived to describe the signal changes seen during DCE-MRI acquisition. The conversion of signal intensity data is not straightforward as the relationship between signal intensity and contrast agent concentration is not linear. Moves have been made in recent years to standardise the various models and associated terms in order to improve reproducibility between groups (Tofts et al 1999). The commonly used kinetic parameters are summarised in *Table I8*. Despite this standardisation, the numerical value of each variable described below will differ for a given arterial input function and tracer kinetic model used. This is a practical limitation in the use of DCE-MRI in multi-centre trials.

Kinetic Parameter	Definition	Recommended for use as primary trial end-point?	Units
IAUC	The initial area under the contrast agent concentration-time curve.	Yes	mM/sec
K^{trans}	Transfer constant reflecting contrast delivery (perfusion) and endothelial permeability.	Yes	Min $^{-1}$
v_e	The volume of extravascular extracellular space per unit volume of tissue.	No	
v_p	The volume of plasma per unit volume of tissue	No	
K_{ep}	Rate constant between the extravascular extracellular space and blood plasma.	No	Min $^{-1}$

Table I8: Standardised terms for commonly used DCE-MRI parameters. Leach et al 2005

IAUC

The initial area under the contrast agent concentration–time curve (IAUC) does not require modelling calculations and so is easy to calculate. The value of IAUC is dependent on the period over which it is calculated; typically this is measured from the point of contrast agent administration to a time 60 seconds after the start time ('$IAUC_{60}$'). It is considered to be reproducible (Evelhoch 1999) and is routinely used as a biomarker in drugs trials. However, the IAUC reading is influenced by several physiological processes, including blood flow, capillary permeability and the EES volume but it cannot differentiate between them. This limits the information that can be gained from this parameter alone (Tofts et al 1999).

***K*^{trans}**

To allow for quantification of the contrast agent kinetics in terms of the physiology of the tumour, it is customary to represent the tissue as containing three compartments (see *figure 3*): the capillary vascular plasma space (v_p), the extravascular extracellular space (EES, v_e) and the intracellular space (v_i) (Tofts et al 1999). Commonly used MRI contrast agents do not pass into the intracellular space but move between the vascular space and EES. The parameters that influence contrast agent distribution (as measured by K^{trans}) are assumed to be restricted to the rate of blood flow and the endothelial permeability-surface area product (PS), accepting that this is a simplified model of the tumour microenvironment. Several models have been applied to clinical trial data to enable calculation of K^{trans} and v_e, many of which are equivalent (Larsson et al 1990, Tofts and Kermode 1991). It is important to note that the interpretation of K^{trans} is dependent on the blood flow to the tumour. When tissue contrast delivery is adequate, K^{trans} reflects the permeability of the capillary membranes. However, when tumour perfusion is limited, K^{trans} represents the blood flow rather than permeability (Tofts et al 1999).

In the studies included in this research, the extended Tofts version of the Kety model (Tofts 1997) was fitted to the DCE-MRI time series at each enhancing tumour voxel using in-house software, as given by

$$C_t(t) = v_p C_p(t) + K^{trans} \int_0^t C_p(t') \exp\left(\frac{-K^{trans}(t-t')}{v_e}\right) dt' \quad (1)$$

where $C_t(t)$ is the concentration of contrast agent at time t in each voxel and $C_p(t)$ is the concentration of contrast agent in the arterial blood plasma (i.e. the arterial input function. This was calculated using a previously published technique (Parker et al 2006). Voxel-wise analysis was performed allowing estimates of the median K^{trans}, median v_p and median v_e.

DCE-MRI parameters as biomarkers

DCE-MRI studies have shown that malignant tumours tend to show faster and higher enhancement levels than are seen in healthy tissue and these differences are reflected in the measured parameters (Padhani 2002). Numerous studies have now shown consistent changes in kinetic parameters following anti-angiogenic therapy (O'Connor et al 2007) but only a small number of trials have established a correlation between DCE-MRI parameters

and clinical benefit. An example is a trial of sorafenib in 44 patients with metastatic renal cell carcinomas, in which patients with high baseline K^{trans} were found to have a better PFS (Hahn et al 2008).

DCE-MRI in metastatic colorectal cancer

DCE-MRI parameters have been used within various early phase trials of novel agents in colorectal cancer. An example of this is the development of the VEGF tyrosine kinase inhibitor vatalanib (PTK787/ZK) in patients with colorectal liver metastases. Phase I trials showed a significant negative correlation between the baseline Ki (bidirectional transfer constant) and increase in vatalanib dose and plasma levels at day 2. Patients with stable disease had a significantly greater reduction in Ki at both day 2 and at the end of the treatment cycle. These findings were used to help to define a biologically active dose of 1000mg for further trials (Morgan et al 2003). More recently, DCE-MRI has been used in a phase I trial of 21 patients with metastatic colorectal cancer treated with the multi-targeted TKI nintedanib. Sixty-seven percent of patients had a ≥40% reduction in K^{trans} and a ≥40% reduction was positively associated with non-progressive tumour status (Mross et al 2014). DCE-MRI has also been used to successfully predict response in 19 patients treated with combination therapy in the form of neo-adjuvant cytotoxic chemotherapy plus bevacizumab for colorectal cancer liver metastases. Progression-free survival benefit was shown for patients with >40% reduction in K^{trans} (DeBruyne et al 2012).

These examples show the potential benefit of DCE-MRI as a biomarker in advanced colorectal cancer. However, to fully understand the impact of combinations of cytotoxic chemotherapy plus anti-angiogenic agents on DCE-MRI parameters, it is crucial to characterise the effects of chemotherapy alone. There is currently limited information on the effect of chemotherapy alone on DCE-MRI parameters in metastatic colorectal cancer. This was explored in one study, in which 23 patients with colorectal liver metastases underwent DCE-MRI at baseline and after 3 cycles of chemotherapy (Vriens et al 2009). This showed an overall significant increase in K^{trans} with treatment but this was not significant on lesion-by-lesion analysis. There was no correlation between any DCE-MRI parameter change and survival. The effect of chemotherapy alone is explored in the paper '*The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer'*, contained within this work.

DCE-MRI in advanced ovarian cancer

A phase I clinical trial of cediranib (AZD2171) in solid tumours demonstrated time-, dose- and exposure-related decreases in $IAUC_{60}$ (IAUC 60 seconds post contrast) (Drevs et al 2007). These changes helped to guide dosages in subsequent phase II trials but the original study was not designed to explore the relationship between kinetic parameter changes and clinical outcome. More recent exploratory analysis of DCE-MRI as a marker of response to single agent bevacizumab in advanced ovarian cancer (n = 14) showed that baseline relative blood flow was not significantly associated with microvessel density, PFS or OS. It did, however, reveal an association between treatment-induced increases in blood flow and PFS at six months (Chase et al 2012).

The study '*Evaluation of dynamic contrast-enhanced MRI for monitoring response to cediranib with cytotoxic therapy in platinum-sensitive ovarian cancer*', contained within this work investigates changes in DCE-MRI parameters patients treated with platinum-based chemotherapy with or without the VEGF TKI, cediranib. The effects of platinum and taxane- based chemotherapy on DCE-MRI parameters in pelvic tumours have been reported in two studies carried out by Lankaster et al. The first showed no effect on IAUC or K^{trans} 24 hours after the first chemotherapy dose (Lankaster et al 2005). The second confirmed no significant change in vascular parameters on DCE-MRI acquired a median of 21 hours after the first dose of chemotherapy (Lankaster et al 2007). They concluded that any changes in these parameters seen in combinations of chemotherapy and anti-angiogenic drugs must be due to the novel agent and not to the chemotherapy.

Future Directions

A 2005 consensus workshop met with aim of providing guidance for the use of DCE-MRI in early phase trials of anti-angiogenic therapies (Leach et al 2005). The guidance stressed the importance of standardised terminology to facilitate the collaboration between groups and the design of multi-centre trials. The emphasis was largely placed on the need for validation of parameters and assessments of reproducibility of imaging techniques. The following points were highlighted as some of the areas to be prioritised for future development:

- Pre-clinical work to define the relationship between MRI parameters and the action of therapeutics.

- Correlation of MRI parameters with physiological information to strengthen the validity of imaging investigations.
- Cross-site standardisation of measurements and analysis to allow for multi-centre trials.

These points form the basis of the design of the studies within this research. The projects aimed to assess pre-treatment and inter-patient variability in functional MRI parameters and to correlate these parameters with physiological markers in the pre-clinical and clinical settings.

DIFFUSION WEIGHTED MAGNETIC RESONANCE IMAGING

Diffusion weighted magnetic resonance imaging (DW-MRI) provides a measure of tissue cellularity, extracellular space tortuosity, and cellular membrane integrity by exploiting the random motion of molecules in liquid (Brownian motion). Cellular membranes impede this random motion and so shorten mean path length of the water molecules. The diffusion time of the molecule can then be derived by calculating the mean path length travelled by protons in the tissue, in a given time period (see *figure I4*). As the cellularity of a tissue alters, so too does the derived parameter of DW-MRI, the apparent diffusion coefficient (ADC). DW-MRI therefore has potential use as a marker of apoptosis in tumour cells.

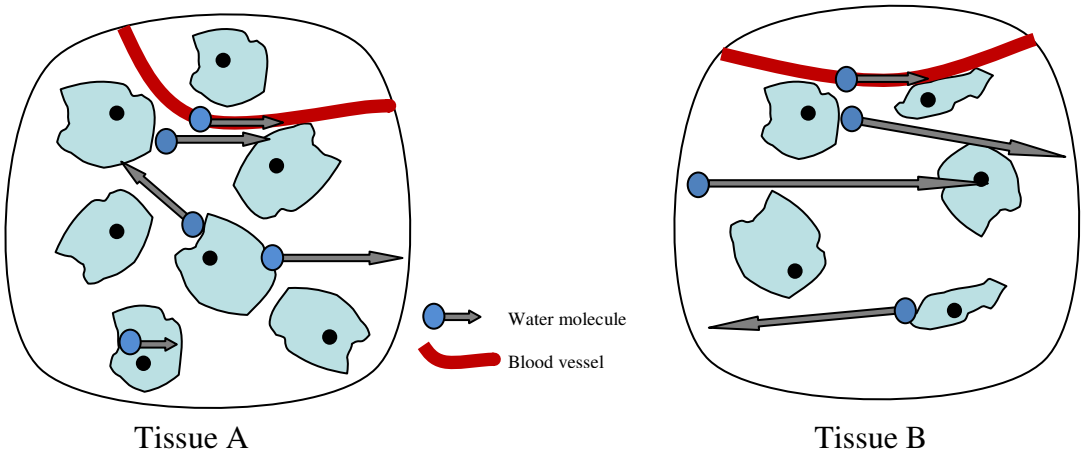

Figure I4: The diffusion of water molecules in tumour tissue
Tissue A: Diffusion is restricted due to high cellularity and intact cell membranes. This results in a low apparent water diffusion (ADC). Note water molecules are seen within extracellular space, intracellular space, and intravascular space, all of which contribute to measured MR signal.
Tissue B: Diffusion is free due to low cellularity and permeable cell membranes. This longer water molecule path length results in a high ADC reading.

The MR signal can be made motion sensitive by the addition of two magnetic field gradients to the signal pulse on T_2-weighted images. The first gradient alters the phase shift of each proton by an amount that is dependent on the distance of the proton from the gradient at the time of the pulse. The second gradient has an equal but opposite effect to the first and so reverses the phase shift of any molecules that have not moved. If the protons have moved between gradient pulses, complete re-phasing will not occur and signal will be lost. This signal loss is proportional to the mean path length of the protons in the same direction as the diffusion gradient and, therefore, to the degree of movement of water. Water movement in fields perpendicular to the gradient would not lead to signal loss. This means that the MR signal is sensitive to both magnitude and direction of water diffusion, which gives information about the architecture of the tissue (see *figure I5*).

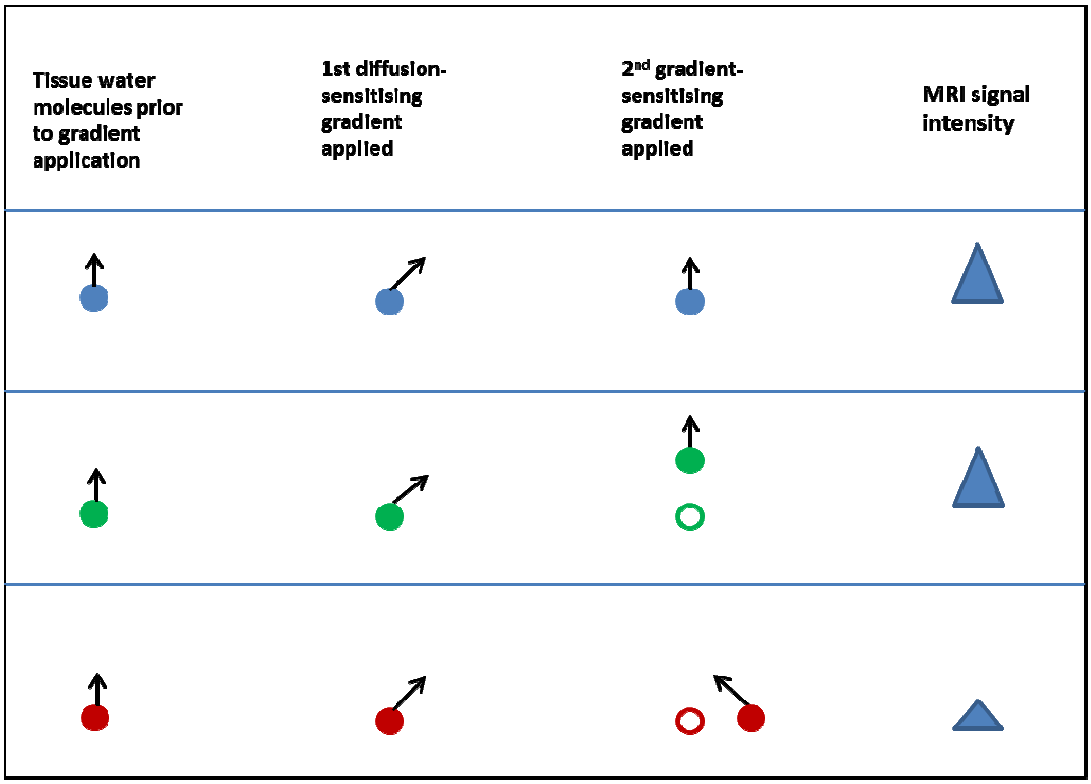

Figure I5: Signal change due to the application of diffusion-sensitising gradients. The water molecules are initially in phase, with the magnetic vectors all pointing in the same direction. The first diffusion gradient dephases all vectors. The second gradient has an opposite and equal effect to the first and so water molecules that have not moved will rephase and lead to no change in signal (blue molecule). Movement perpendicular to the gradient does not cause a drop in signal intensity, as the molecule is rephased by the second gradient (green molecule). Water molecules that move in the direction of the gradients will be incompletely rephased (red molecule) and so this movement causes a drop in signal intensity, which is measured in DW-MRI techniques.

The weighting of the diffusion gradients is indicated by the *b* value, which can be adjusted by altering the gradient pulse amplitude; gradient duration or the time between the paired gradients. Application of these gradients results in signal losses in proportion to the weighting of the gradients. The signal losses are used to calculate the apparent diffusion coefficient (ADC), which is used as the measurable parameter in clinical diffusion weighted imaging. In highly cellular tissue, ADC is low as the movement of extracellular water is restricted whereas in cystic tumours, movement will be increased and the ADC higher. The distances of movement measured are clearly extremely small, making DW-MRI images prone to artefact from motion in surrounding tissue. This presents particular

challenges when imaging areas close to moving structures such as great vessels and the diaphragm. At lower *b* -values (<50-100 s/mm^2) calculated ADC will be affected by tumour blood flow, whereas at higher *b* -values bulk water movement contributes less to ADC and ADC will approximately reflect the degree of water diffusion in the extravascular extracellular space. However, the overall signal-to-noise ratio is worse and image distortion is greater with high *b* values (Patterson et al 2008).

DW-MRI parameters as biomarkers

DW-MRI imaging is non-invasive; requires no contrast agents; does not use ionizing radiation and can be acquired relatively quickly. These characteristics make it an appealing method of monitoring response to treatments, where multiple scans are likely to be required. Malignant tumours generally have lower ADC values than healthy tissues. This is likely to be related to a combination of increased cellularity, increased tortuosity in the extracellular space and disorganisation in the malignant tissues. In most malignant tumours, successful treatment is reflected by increases in ADC.

Most work with DW-MRI in oncology was initially focused on brain tumours. In 2000, an animal study evaluating gliomas in rats showed that mean tumour ADCs correlated with changes in tumour cellularity. The study then went on to investigate the feasibility of serial scans in the clinical management of patients with primary brain tumours. It concluded that increased diffusion values could be detected in human brain tumours shortly after treatment initiation and that degree of diffusion changes corresponded with clinical outcome. The hope was that ADC could eventually be used as an early surrogate marker for treatment response (Chenevert et al 2000). Subsequently, a small clinical cohort of 28 patients with glioblastoma multiforme underwent DW-MRI after surgery but before radiotherapy. This revealed a prolonged median survival time in patients with a high mean ADC value compared to those with low mean ADC value (21.7 v 11.2 months, respectively, P = 0.004) (Oh et al 2004). Further investigation into extra-cranial sites of disease is ongoing with the continued hope of validating these potential biomarkers.

DWI-MRI in colorectal cancer

Previous studies of DW-MRI in primary rectal tumours have demonstrated the use of DW-MRI to predict response to chemotherapy and chemoradiation (Dzik-Jurasz et al 2002; DeVries et al 2003: Sun et al 2010). However, at the time of designing the current studies, data were limited on the use of DW-MRI in metastatic colorectal cancer. Advances have been made over the last few years and the results of these studies are summarised in *Table I9.*

The majority of the studies demonstrate a significant relationship between pre-treatment ADC and response to treatment, in which high baseline ADC predicts for poor response to treatment, as defined by RECIST criteria. The cause for this relationship has not yet been fully elucidated, but it is hypothesised that high baseline ADC readings may be due to tumour necrosis, which may be characteristic of an aggressive tumour phenotype; poor perfusion and a hypoxic, acidic tumour microenvironment. This is known to reduce chemotherapy delivery and may, therefore, reduce tumour response.

Study	MRI protocol	Agents studied	Scan schedule	Key results
Koh et al. 2007 n = 20	1.5T *b* = 0,150, 500 s/mm^2	Oxaliplatin + capecitabine 6 cycles	Pre-treatment and within 3 weeks post chemotherapy	High pre-treatment mean ADC was predictive of poor response. Responding lesions had a significant increase in mean ADC with chemotherapy, whereas non-responders did not.
Cui et al. 2008 n = 23	1.5T *b* = 0 and 800 s/mm^2	Chemo-therapy (various regimens)	Pre-treatment and at days 3, 7 and 42 after commencing treatment.	High pre-treatment mean ADC was predictive of poor response. Responding lesions had a significant increase in mean ADC with chemotherapy at days 3 and 7, whereas non-responders did not.
Heijmen et al 2012 n = 19	1.5T *b* = 50, 300 and 600 s/mm^2	Pre- liver resection baseline scans	Two baseline scans prior to liver resection.	ADC values were significantly related to the proliferation index Ki67 and BCL-2 expression in the metastases.
Anzeidi et al 2011 n = 18	1.5T *b* = 0,50,500 s/mm^2	Oxaliplatin + capecitabine + bevacizumab	Pre-treatment and 6 months post treatment	Pre-treatment ADC was not predictive of response Responding lesions had a significant increase in mean ADC with chemotherapy, whereas non-responders did not.
Tam et al 2013 n = 102	1.5T *b* = 0,50,500 breath hold or *b* = 0,50, 100,250,500, 750 free breathing	Chemo-therapy (various regimens)	Retrospective analysis of pre-treatment DW-MRI scans.	High pre-treatment mean ADC was predictive of poor response. Baseline ADC values did not predict for PFS or OS.

Table I9: DW-MRI as a parameter in colorectal hepatic metastases: a summary of current published data.

The literature also describes an increase in ADC with treatment in those tumours which subsequently respond to treatment, which is not seen in non-responding tumours. This may occur as tumours respond by becoming less cellular. This is supported by the finding that a significant association exists between ADC values and both the proliferation index Ki67 and BCL-2 expression in CRC metastases (Heijmen et al 2012). Tam et al (2013) were the first group to explore the relationship between pre-treatment ADC and patient outcome in this setting. This large retrospective study did not demonstrate a significant relationship between baseline ADC and survival but recommended that this be a focus of future prospective studies. A recent study of 39 patients undergoing first-line chemotherapy for mCRC found that pre-treatment ADC was a significant predictor of both PFS and OS but that on-treatment change in ADC was not related to outcome (Heijmen et al 2015).

DW-MRI in ovarian cancer

In advanced ovarian cancer, diffusion weighted MRI sensitivity and specifically detects peritoneal implants and is used to improve staging accuracy when added to conventional MRI techniques (Low et al 2009). More recently, Kyriazi et al sought to explore the use of ADC histogram analysis to evaluate response to platinum or taxane based chemotherapy in the neoadjuvant treatment of advanced ovarian cancer. Forty-two patients underwent DW-MRI scanning at baseline and again after cycles one and three of various chemotherapy regimens. Pre-treatment mean ADC was not predictive of response but responders had a higher ADC after cycle one and after cycle three than non-responders. Skew and kurtosis of the ADC distribution histograms were significantly altered by cycle three in the responders but not in non-responders, providing an early marker of response (Kyriazi et al 2011). This was explored in a study of 22 patients who underwent scans at baseline and following three cycles of neo-adjuvant platinum based chemotherapy for advanced ovarian cancer. Interestingly, significant increases in ADC were seen in responding ovarian tumours but not in peritoneal or omental metastases. They also reported that baseline mean ADC was significantly lower in peritoneal tumour than in ovarian or omental deposits, suggesting biological heterogeneity within the same patient (Sala et al 2012).

Future Directions

A 2009 consensus statement on the use of DW-MRI as a cancer biomarker outlined the following areas for future development (Padhani et al 2009):

- DW-MRI should be tested as an imaging biomarker within well-defined clinical trials, particularly those including tissue sampling or survival indicators.
- Baseline reproducibility studies should be part of study design.
- Validation and standardisation is urgently needed to exploit the potentially valuable role of DW-MRI and this will require collaboration between groups.

Diffusion weighted MRI appears to be a promising tool in predicting response to chemotherapy in metastatic colorectal cancer. The next steps for validation include studies using survival outcomes in combination with blood and other imaging biomarkers. This forms the basis of the study of DW-MRI in metastatic cancer ('*The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer')* and in the preclinical setting ('*ADC biomarkers detect caspase induced apoptosis in HT29 colorectal xenografts')*. There are few studies addressing the use of ADC as a biomarker in ovarian cancer, particularly in the setting of targeted therapy. Exploration of these techniques forms the basis of the study of DW-MRI in ovarian cancer ('*Evaluation of dynamic contrast-enhanced MRI for monitoring response to cediranib with cytotoxic therapy in platinum-sensitive ovarian cancer*').

OVERVIEW OF STUDIES

PhD project aims

This PhD thesis consists of three separate studies; all of which aim to evaluate the use of functional MRI techniques as prognostic and/or predictive biomarkers of angiogenic or apoptotic pathways in advanced cancer. This thesis is presented in an alternative format to incorporate the three separate studies that were carried out to explore this common aim. The thesis is constructed around these three studies, which are written as journal papers.

PROJECT ONE: The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer

This study sought to explore the effect of standard chemotherapy on a panel of MR imaging and circulating markers of angiogenesis and apoptosis in colorectal cancer liver metastases. It is crucial to understand how these parameters behave in this setting before the biomarkers can be confidently used in trials of dual therapy, in which targeted agents are added to the chemotherapy regimen. Twenty patients with liver metastases from colorectal cancer treated with oxaliplatin and 5-fluorouracil (5-FU) or capecitabine chemotherapy in the first-line setting underwent DCE-MRI to extract measurements of tumour vascular parameters and DW-MRI scans to extract measurements relating to cell density. Tumour volume measurements were also obtained from MRI and blood was collected for analysis of circulating markers of angiogenesis, cell death and CTCs.

My input

I designed and wrote the clinical protocol, patient information leaflet and consent form for this study and submitted the documentation for local Research and Development approval as well as Research Ethics Committee approval. I trained clinical staff in recruitment and consent for the study and acted as study co-ordinator during recruitment and follow up. I recruited and consented patients; clinically assessed for adverse events; performed venepuncture for trial blood tests and supervised MRI scans. I carried out all analysis of blood for circulating markers of angiogenesis using Multiplex ELISAs and for circulating CK18 using single plex ELISAs. I carried out all statistical analysis of circulating markers, CTC and MRI data and wrote and submitted the clinical paper for journal submission.

PROJECT TWO: Diffusion weighted magnetic resonance imaging biomarker detection of apoptosis in colorectal cancer xenografts

This study evaluated the potential of DW-MRI to detect and quantify apoptosis in a specifically engineered model of apoptosis, *in vivo*. DW-MRI was used to evaluate apoptosis in HT29 colorectal cancer xenografts that had been engineered to express constitutively activated caspase 3 in an inducible manner, referred to as the "death switch" Srinivasula et al 1998). This model exploits the convergence of the intrinsic and extrinsic caspase signalling pathways on caspase-3 to produce synchronous apoptosis. Induction of apoptosis with doxycycline leads to tumour regression; activation of IHC markers of apoptosis and release of established circulating biomarkers of cell death. Thus this model offers a unique opportunity to investigate the potential of ADC to detect apoptosis over time and to compare changes in ADC with expected changes in IHC and circulating biomarkers under controlled experimental conditions. In the experiments described in this paper, DW-MRI scanning was carried out 6 and 24 hours after induction of the apoptotic cascade with doxycycline and both tumours and blood were taken for analysis of markers of cell death.

My input

I designed and wrote the pre-clinical protocols for these studies and co-ordinated the studies including involvement with animal care. I cultured cells for implantation and carried out all MRI scanning. I analysed all blood for CK18, including validation experiments, and analysed the tumours using IHC techniques. I was involved with the quality analysis process of MR analysis and responsible for all statistical analysis of CK18, IHC and MRI results. I wrote the manuscript for submission.

PROJECT THREE: Evaluation of dynamic contrast-enhanced MRI for monitoring response to cediranib with cytotoxic therapy in platinum-sensitive ovarian cancer.

Companion sub-protocol to MRC sponsored
ICON6: Randomised double-blind phase III trial of cediranib (AZD 2171) in relapsed platinum sensitive ovarian cancer.

This project evaluates DCE-MRI and DW-MRI parameters in the setting of dual therapy with chemotherapy and a VEGF inhibiting drug, this time in patients with advanced ovarian cancer. The use of these agents is rapidly developing and robust biomarkers must be validated to aid this development. Ten patients who were participating in the phase III ICON6 trial were included in this sub-study, which investigated the potential role of DCE-MRI and DW-MRI techniques to provide biomarkers in patients treated with chemotherapy and cediranib. The ICON6 trial investigated the benefit of administering cediranib with cytotoxic chemotherapy and as maintenance therapy in patients with relapsed platinum-sensitive epithelial ovarian cancer (Ledermann et al 2013 and Ledermann et al 2016). Patients who participated in the imaging sub-study had two pre-treatment DCE-MRI and DW-MRI scans within 2 weeks, followed by scans on cycle 1 day 2 and cycle 2 day 2.

My input

I amended the clinical protocol, patient information leaflet and consent form for this study and submitted the documentation for local Research and Development approval as well as Research Ethics Committee approval, including collaboration with a second recruitment site. I acted as study co-ordinator during recruitment and follow up. I recruited and consented patients; clinically assessed for adverse events and supervised MRI scans. I carried out all statistical analysis of MRI data and wrote the clinical paper for journal submission.

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METHODS

The following section expands on the methods described within the three journal papers within this thesis.

***Multiplex ELISA method** (Aushon Biosystems, Boston, USA)*

Blood samples for measuring angiogenic markers were drawn directly into 6ml EDTA vacutainers and plasma was separated from the blood cells by centrifugation of the sample at 3000*g* for 10 minutes. Enzyme-Linked Immuno-Sorbent Assays (ELISAs) were performed using SearchLight chemiluminescent arrays and SearchLight Plus charged couple device imaging system (*Aushon Biosystems, Boston, US*). These are multiplex assays that can be used for the quantification of up to six different proteins in each well of a ninety-six well plate. This multiplex approach allowed a greater number of analytes to be investigated using a small blood volume. All assays were performed at the Clinical and Experimental Pharmacology GCPL laboratories, Cancer Research UK: Manchester Institute and were subject to in-house validation as previously described (Backen et al 2009). Briefly, 50 µl calibration standards or samples were added in duplicate to the 96-well ELISA plate which was covered with an adhesive lid and incubated for 1 hour at room temperature shaking throughout at 200 rpm. At the end of the incubation, the plate was washed three times with kit wash buffer. 50 µl biotinylated antibody reagent was added to each well of the plate which was covered with an adhesive lid and incubated for 30 min at room temperature shaking at 200 rpm. The plate was washed three times. 50 µl streptavidin-HRP reagent was added to each well of the plate, which was then incubated for 30 min at room temperature shaking at 200 rpm. The plate was washed three times. 50 µl SuperSignal substrate was added to each well of the plate which was then imaged in the charge-coupled device (CCD) camera within 10 minutes from the addition of the signal substrate. The light signal for each spot is proportional to the concentration of the target protein to allow for quantification of the protein. The upper and lower limits of detection were set at the highest and the lowest concentrations within the standard curve.

Protein concentrations were assessed using six-plex ELISAs of Ang2, FGFb, HGF, PDGFbb, VEGFA and VEGFC and IL6, IL8, KGF, PlGF, VEGFR1, VEGFR2; duplexes of Ang1 and Tie2 and E-selectin and VCAM-1 and single plexes of VEGFD and SDF1b.

M65 ELISA (*Peviva, Sweden*)

Blood samples were collected using standard phlebotomy techniques, using a vacutainer system (*BD Franklin Lanes, NJ*), into EDTA tubes. Blood samples were centrifuged at 2000 g for 10 min and serum was collected by pipette. Serum was aliquotted for storage at -80^{0}C and thawed immediately prior to analysis. The M65 ELISA assay is a commercially available kit based on a 96-well plate format and analysis was carried out according to the manufacturer's instructions, previously described (Cummings et al 2005). In brief, 25 µl of sample was added to each well, which was coated with a mouse monoclonal antibody that binds to an epitope on CK18. 75 µl of HRP-conjugated monoclonal antibody solution was added to act as the detection antibody. Samples were then incubated for 2 hours at room temperature with constant shaking, after which excess unbound conjugate was removed using wash solution. Colour development was achieved by dark incubation with 200 µl of 3,30,5,50-tetramethyl-benzidine solution for 20 min. The reaction was stopped with 50 µl of 1.0 M sulphuric acid and absorbance was measured in a microplate reader, within 30 minutes. By plotting a standard curve of known concentrations of M65 antigen standards versus absorbance, the amount of antigen in the quality control and serum samples was calculated. In the case of the pre-clinical samples of mouse serum, the technique described above was used, with the addition of heterophilic blocking reagent (HBR) (*Scantibodies, USA*) to overcome the problem of heterophilic antibody interference in the immunoassay.

M65 Epideath ELISA(*Peviva, Sweden*)

Many of the pre-clinical samples of mouse serum proved to have CK18 concentrations above the dynamic range of the M65 Assay, and so the M65 Epideath Assay was used to re-analyse all pre-clinical samples. This assay works on the same principles as the M65 Assay but has a wider range of detection. HBR plus (*Scantibodies, USA)* was added to the samples, following in-house validation of this reagent. XG4 human myeloma carcinoma cells were treated with 1uM staurosporin for 24 hours and the supernatant was then harvested for spiking. As this supernatant is CK18 rich, it was used to provide known concentrations of CK18 in the samples by spiking the supernatant into fetal calf serum and measuring the M65 Epideath results, as per manufacturer's instructions. Various HBR reagents (HBR plus, HBR1, 3, 6, 9, 11 from the *Scantibodies Assay Development Blocking Kit, USA*) were then added to the samples and the assay was re-run. This revealed HBR plus to be effective in blocking heterophilic antibody interference without altering the measurements of known concentrations of CK18, meaning that this reagent was used for

all mouse serum experiments. The M65 Epideath assay was run as described above but with an initial incubation period of 4 hours, rather than 2 hours.

M30 Assay *(Peviva, Sweden)*

Again, the M30 Assay is a commercially available kit and was carried out following the manufacturer's instructions, with the same method as that described above, but with a 4 hour initial incubation period rather than 2 hours.

CTC enumeration method (*Janssen Diagnostics, UK*).

Sample processing and analysis were carried out according to the manufacturer's instructions. Ten millilitres of blood were drawn into a CellSave preservation tube and CTC analysis was carried out within 96 hours, using the FDA-approved CellSearch technology tube. The CellSearch CellTracks AutoPrep System was used to prepare the samples and the Cell Search CellTracks Analyzer II CellTracks used to image the cells. The CellSearch CTC Kit contained ferrofluid particles coated with antibodies to epithelial cell adhesion molecule (EpCAM) to immunomagnetically separate cells of epithelial origin from blood. After immunomagnetic capture and enrichment, cells are fixed and fluorescent reagents added to identify and enumerate CTCs. The fluorescent reagents include anti-CK-Phycoerythrin (PE) specific for the intracellular protein cytokeratins 8, 18 and 19 (characteristic of epithelial cells), 4, 6-diamidino-2-phenylindole (DAPI), which stains the cell nucleus, and anti-CD45-Allophycocyanin (APC) to identify and exclude leucocytes. Sample analysis is performed by the CellTracks Analyzer II, a four colour automated fluorescence microscope that scans the surface of the cartridge, acquires images and displays any CK-PE and DAPI positive cells to the user in a gallery format. Final classification is user-dependent. Cells were classified as CTCs if they had morphological features consistent with that of a tumour cell; were CD45 negative and positive for DAPI staining and cytokeratins.

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The impact of cytotoxic chemotherapy on imaging and circulating biomarkers of angiogenesis in colorectal cancer

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ABSTRACT

Background

Patients with metastatic colorectal cancer often achieve partial response to first-line chemotherapy but subsequent resistance to treatment limits the overall survival (OS) of this patient group. Magnetic resonance imaging (MRI) and circulating markers of angiogenesis and apoptosis have been developed to allow treatment decisions to be made early, thus reducing treatment related toxicities and cost. However it is crucial to characterise fully the chemotherapy-related changes in these parameters to allow interpretation of clinical trial data when chemotherapy is used in combination with novel molecularly-targeted agents. This study sought to explore the effect of standard chemotherapy on a panel of imaging and circulating markers of angiogenesis and apoptosis in colorectal cancer liver metastases.

Method

Twenty patients with liver metastases from colorectal cancer treated with oxaliplatin and 5-Fluorouracil (5-FU) or capecitabine chemotherapy in the first-line setting underwent dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) to extract measurements of tumour vascular parameters and diffusion weighted MRI (DW-MRI) scans to extract measurements relating to cell density. Tumour volume measurements were also obtained from MRI. Blood was collected for analysis of circulating markers of angiogenesis, apoptosis and circulating tumour cells (CTCs). The scans and blood collection were scheduled prior to treatment and on cycle 1 day 2, cycle 1 day 8, cycle 2 day 2 and following 6 cycles of chemotherapy.

Results

The MRI-determined whole tumour volume (WTV), fell significantly in all tumours by chemotherapy cycle 2 day 2 (C2D2) and reduced further in all tumours by cycle 6. By C2D2, the cohort DCE-MRI-derived iAUC, K^{trans} and v_e were significantly higher than before treatment and remained elevated after cycle 6. Patients with a K^{trans} higher than pre-treatment levels at C2D2 had an overall survival of 10.1 months whereas the equivalent statistic was 22.1 months in those without a significant rise at this time-point ($p = 0.049$). A positive association existed between MRI derived WTV and CTC number at the pre-treatment time-point ($p = 0.005$). A negative correlation was found between K^{trans} and CTC number before treatment ($p = 0.042$) and at C1D2 time-point ($p= 0.018$). Chemotherapy led to detectable dynamic changes Ang1, FGFb, IL8, PDGFbb, VEGFA, VEGFC,

VEGFR2 (down) and VEGFD and VCAM1 (up). Pre-treatment FGFb and VEGFC plasma concentrations correlated positively with progression-free survival (PFS) (VEGFC r= 0.56, p = 0.01; FGFb r = 0.50, p= 0.03). The median apparent diffusion coefficient (ADC) in this cohort significantly increased by C2D2 (p = 0.004) and C6 (p = 0.005).

Conclusions

This study demonstrated that significant early changes in MRI and circulating angiogenic biomarkers followed chemotherapy treatment alone, reflecting chemotherapy-induced alterations to the tumour vasculature and cellularity. This must be taken into account when interpreting results of studies using targeted agents alongside these chemotherapy regimens. A rise in K^{trans} by cycle 2 day 2 of chemotherapy correlated with a shorter OS which may be a useful biomarker of response. For the first time, a negative relationship between CTCs and K^{trans} has been observed. Further evaluation of these techniques is warranted with the aim of creating a panel of biomarkers which will be predictive for treatment outcome.

INTRODUCTION

Colorectal cancer is common in the western world with 33,765 new cases registered in the UK in 2013 (Office of National Statistics 2013). Survival is poor in patients with metastatic disease; only 8% survive beyond five years from diagnosis (CRUK 2015). Efforts are focused currently on improving outcomes by adding novel agents targeting angiogenic and apoptotic pathways to the backbone of conventional cytotoxic chemotherapy (Khan et al 2013). Clinically meaningful biomarkers, such as those provided by functional MRI, are needed to accelerate the development of these drugs by identifying who will benefit from these targeted agents. It is crucial that we fully characterise the effects of chemotherapy on potential biomarkers before we can use them to build combination treatments.

DCE-MRI techniques have been used extensively in early phase clinical trials of drugs targeting the vascular endothelial growth factor (VEGF) pathway (O'Connor et al 2007). The parameters derived from these images give information regarding the dynamic changes in the tumour microvasculature associated with inhibition of VEGF. There is currently only limited information on the effect of chemotherapy alone on DCE-MRI parameters in metastatic colorectal cancer. This has only been explored in a single study of 23 patients with colorectal liver metastases who underwent DCE-MRI at baseline and after 3 cycles of chemotherapy (Vriens et al 2009). This demonstrated an overall significant increase in K^{trans} that was observed with treatment but was not significant in lesion-by-lesion analysis. There was no correlation between any DCE-MRI parameter change and survival outcomes.

The relationship between CTCs and several DCE-MRI parameters was investigated to assess whether the measurements provide independent useful information. CTCs are present in the blood of cancer patients but are rare in healthy volunteers (Allard 2004). The number of CTCs detected can be used as a prognostic marker in metastatic CRC and enumeration technology platform (Cellsearch) has been FDA-approved for use in metastatic CRC (Cohen et al 2008).

The use of multiplex ELISAs for measurement of circulating proteins in plasma has been previously developed and validated (Backen et al 2009). This study explored the effect of chemotherapy on the pre- and post- treatment plasma concentrations of 18 angiogenesis-

related proteins and the relationships between these proteins and DCE-MRI parameters. These were vascular endothelial growth factors A, C and D and the receptors R1 and R2 (VEGFA); VEGFC; VEGFD (Werther et al 2000); VEGFR1 (Duda et al 2010); VEGFR2 (Drevs et al 2007); fibroblast growth factor (FGFb) (Abdel-Rahman 2015); interleukins 6 and 8 (IL6) (Willett et al 2009); IL8; angiopoietin 1 (Ang1) (Satoh et al 2008); angiopoietin 2 Ang2 (Hashizume et al 2010); tunica internal endothelial cell kinase 2 (Tie2) (Backen et al 2014); placental growth factor (PlGF) (Van de Veire et al 2010); platelet-derived growth factor (PDGFbb) (Braicu et al 2013); hepatocyte growth factor (HGF) (Yonesaka et al 2015); keratinocyte growth factor (KGF) (Narita et al 2009); stromal cell-derived factor 1b (SDF1b) (Kollmar et al 2007); endothelial-leucocyte adhesion molecule 1 (E selectin)(Wittig et al 1996) and vascular cell adhesion molecule 1 (VCAM1) (Alexiou et al 2001).

DW-MRI is a technique that can be used to characterise the cellularity of a tissue. The key derived parameter from of DW-MRI, the apparent diffusion coefficient (ADC), has potential as a marker of apoptosis in tumour cells. The majority of previous studies demonstrate a significant relationship between pre-treatment ADC and response to treatment, in which high baseline ADC predicts for poor response to treatment, as defined by RECIST (Koh 2007, Cui 2008, Anzidei 2011). However, a large retrospective study of patients with colorectal cancer liver metastases did not detect any significant relationship between pre-treatment ADC and survival (Tam 2013).

The M65 ELISA has been validated previously for detection of total circulating cytokeratin 18 (CK18), and is a marker of cell death (Cummings et al 2006, Greystoke et al 2008). High pre-treatment circulating CK18 concentrations are predictive of poor response to treatment and to shorter OS times in metastatic colorectal cancer (Greystoke et al 2012, Koelink et al 2009).

This study sought to explore the effect of standard chemotherapy on a panel of imaging and circulating markers of angiogenesis and apoptosis in colorectal cancer liver metastases.

Patients and Methods

This trial was performed with local ethics committee approval in accordance with the UK Clinical Trials regulations for compliance with Good Clinical Practice Guidelines (NRES Committee North West, Liverpool East, 11/NW/0118) (*The Medicines for Human Use (Clinical Trials) Regulations 2004*). Local research and development department approval was obtained and laboratory work was carried out in accordance with the principles of Good Clinical Laboratory Practice (World Health Organization 2009).

Patients

This was a prospective, single-centre biomarker study at The Christie NHS Foundation Trust (Manchester, UK). From October 2011 to November 2013, 20 patients were recruited with a new diagnosis of metastatic colorectal cancer, treated with oxaliplatin and 5-Fluorouracil (5-FU) or capecitabine chemotherapy in the first-line setting (Cheeseman 2002, Cassidy 2004). Inclusion criteria were; histopathologically confirmed metastatic colorectal cancer, metastases measuring at least 3cm in longest diameter (for MRI purposes), patients due to commence first line conventional oxaliplatin and 5FU or capecitabine chemotherapy, considered fit for cytotoxic chemotherapy by the treating oncologist with WHO performance status 0-2, white cell blood count greater than $4x10^9$ /L, platelet count greater than 100 $x10^9$ /L, serum bilirubin concentration less than 1.25×upper limit of normal, alkaline phosphatase concentration less than 5×upper limit of normal, calculated glomerular filtration rate greater than 50 mL per min, older than 18 years and able to give informed consent. The exclusion criteria were standard MR scanning exclusion criteria relating to metal implants; known allergy to MRI intravenous contrast, inability to comply with the trial protocol, use of adjuvant chemotherapy within the previous twelve months, history of other malignancies within previous five years, use of any investigational product with chemotherapy treatment; conditions in which research blood sampling may significantly increase the risk of complications to the patient or the investigator, pregnant or breast-feeding women and fertile woman of childbearing potential not using adequate contraception.

Treatment

The treatment followed the standard protocol for first-line chemotherapy at the time the trial was commenced. Patients were treated with oxaliplatin and 5-FU (oxaliplatin 85mg/m^2; folinic acid 350mg and 5-FU 400mg/m^2 on day one then 5-FU 2400mg/m^2 46hr IV infusion on a two-weekly cycle) or oxaliplatin and capecitabine (oxaliplatin 130mg/m^2 on day one and capecitabine 1000mg/m^2 on day one to fourteen of a three-weekly cycle). Treatment response was assessed using computerised tomography (CT) scans before treatment, after 12 weeks of chemotherapy, after 24 weeks of chemotherapy and as indicated by clinical signs of disease progression. Treatment response was evaluated according to RECIST v1.1 (Eisenhauer et al 2009). MRI-specific toxicities were recorded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.4 (National Cancer Institute 2009).

MRI Acquisition

Pre-treatment MRI scans were performed twice before commencing chemotherapy, a minimum of 24 hours apart, to assess the reproducibility of measurements and thus the impact of treatment on imaging parameters. The same protocol was then repeated on cycle 1 day 2; cycle 1 day 8, cycle 2 day 2 and after 12 weeks of treatment, where cycle 1 day 1 was defined as the day of the first chemotherapy dose. DW-MRI and DCE-MRI were performed in the same scanning session.

Imaging was performed using a 1.5 T Philips Achieva scanner (*Philips Healthcare, Best, The Netherlands*) at the Wolfson Molecular Imaging Centre, University of Manchester. DCE-MRI was carried out as previously described (O'Connor et al 2011). The field of view (FOV) was centred on the liver. In each examination, T_1-weighted fast field echo images (TR=10 ms, TE=4.6 ms, α=15°) and T_2-weighted single shot turbo spin echo images (TR=606.5 ms, TE=80 ms, α=90°) were acquired. Both sequences employed FOV 375 × 375 mm^2, matrix 256 × 256 with a 4-mm slice thickness.

For the DCE-MRI series, 75 3D axial volumes were acquired consecutively (TR=4.0 ms, TE=0.82 ms, α=20°, one signal average, FOV of 375 × 375 mm^2, matrix 128 × 128; in-plane voxel size 2.93 × 2.93 mm^2) following calculation of baseline T_1 using the variable flip angle method (Fram et al 1987) (α=2°/10°/20°; four signal averages; identical TR, TE, imaging matrix, and slice thickness). Temporal resolution was 4.97 s During the dynamic

sequence, 0.1 mmol/kg of gadoterate meglumine contrast agent (*Dotarem, Guebert, France*) was administered intravenously at the sixth dynamic time-point at a rate of 3 ml/s, using a Medrad Spectris power injector (*Bayer Healthcare Pharmaceuticals, USA*). DW-MR images were acquired using a non-breath holding, fat-suppressed pulsed gradient spin-echo (PGSE) echo-planar imaging sequence with the following parameters: FOV 375 mm x 375 mm; slice thickness 4 mm; number of slices 25; matrix 256 x 256; in-plane resolution 1.46 mm x 1.46 mm; TR 3416 ms; TE 90 ms with *b* values of 0, 150, 500 and 800 s/mm².

MRI Analysis

Regions of interest (ROIs) were defined manually by an experienced operator (YW) for the WTV using Java Image software (*JIM version 5.0, Xinpase Systems Ltd, UK*) and with reference to the T_1- and T_2-weighted images as well as the DCE-MRI images. The ROIs were chosen if they fulfilled the inclusion criteria (liver metastases of at least 3cm). The ROI was re-calculated for each scan, with reference to the pre-treatment scans. The arterial input function (AIF) was determined for each patient visit using an automated technique in the nearest feeding artery (REF) (Parker et al 2003). An assumed haematocrit of 0.42 was input into the modelling process. Output parameters included whole tumour volume (WTV), tumour enhancing volume, tumour enhancing fraction, and tumour median values of the initial area under the DCE-MRI contrast agent concentration time course at 60 s ($iAUC_{60}$), the endothelial contrast agent transfer coefficient (K^{trans}), fractional blood plasma volume (v_p), and the fractional extravascular extracellular volume (v_e), as derived using the extended Kety model (Tofts 1997). Pre-treatment 'whole tumour permeability' was also calculated (WTV x K^{trans}) to adjust for the bulk of disease. All DCE-MRI analyses were performed using in-house software, Manchester Dynamic Modelling (MaDyM). DW-MRI was analysed by fitting the tumour ROI data to obtain the water apparent diffusion coefficient (ADC). Voxel-wise estimates of ADC were calculated by fitting the image data to $S(b) = S_0e^{-b*ADC}$, where *b* is the *b* value for each DWI acquisition, using in-house written software. ADC maps were generated and median tumour ADC was calculated to summarise each tumour.

Circulating Tumour Cells

Whole blood was drawn for enumeration of CTCs at time-points corresponding to the MRI scanning schedule. The aim was to test the hypothesis that vascular changes seen on DCE-MRI correlate with the number of tumour cells released into the circulation. Ten millilitres of blood were drawn into a CellSave preservation tube and CTC analysis was carried out within 96 hours, using the FDA-approved CellSearch technology tube (*Janssen Diagnostics, UK*). The CellSearch CellTracks AutoPrep System (*Janssen Diagnostics, UK*) was used to prepare the samples and the Cell Search CellTracks Analyzer II CellTracks (*Janssen Diagnostics, UK*) used to image the cells. The CellSearch CTC Kit (*Janssen Diagnostics, UK*) contained ferrofluid particles coated with antibodies to epithelial cell adhesion molecule (EpCAM) to separate cells of epithelial origin immunomagnetically from blood. Leucocytes were excluded by using a fluorescent antibody to CD45. Epithelial cells were identified through antibodies to cytokeratins 8, 18 and 19 and a nuclear dye, 4, 6-diamidino-2-phenylindole (DAPI) (Kagan et al 2012). Cells were classified as CTCs if they were CD45 negative and positive for DAPI staining and cytokeratins. Sample processing and analysis were carried out strictly according to the manufacturer's instructions.

Soluble angiogenesis-associated proteins

Blood was drawn for quantification of soluble angiogenesis-associated proteins at time-points corresponding to the MRI scans to test the hypothesis that vascular changes seen on DCE-MRI would correlate with the concentration of circulating angiogenic factors. Blood samples for measuring angiogenic markers were drawn directly into 6ml EDTA vacutainers and plasma was separated from the blood cells by centrifugation of the sample at 3000*g* for 10 minutes. Enzyme-Linked Immuno-Sorbent Assays (ELISAs) were performed using SearchLight chemiluminescent arrays and SearchLight Plus charged couple device imaging system (*Aushon Biosystems, Boston, US*). These are multiplex assays that can be used for the quantification of up to six different proteins in each well of a ninety-six well plate. This multiplex approach allowed a greater number of analytes to be investigated using a small blood volume. All assays were performed at the Clinical and Experimental Pharmacology GCPL laboratories, Cancer Research UK: Manchester Institute and were subject to in-house validation as previously described (Backen et al 2009). Protein concentrations were assessed using six-plex ELISAs of Ang2, FGFb, HGF,

PDGFbb, VEGFA and VEGFC and IL6, IL8, KGF, PlGF, VEGFR1, VEGFR2; duplexes of Ang1 and Tie2 and E-selectin and VCAM-1 and single plexes of VEGFD and SDF1b.

Circulating markers of cell death

CK18 is a member of the intermediate filament family of cytoskeletal proteins and is widely expressed in epithelial and endothelial cells. The M65 ELISA measures total soluble CK18 which is released during apoptotic and necrotic forms of cell death. M65 has been validated as a marker of cell death in colorectal cancer (Greystoke et al 2012) and was examined in this study alongside the diffusion weighted imaging data, to explore the relationship between circulating and imaging biomarkers of cell death. Blood was collected in tubes containing a silica clot activator and centrifuged at 2000*g* for 10min to obtain serum for analysis. The M65 ELISA assay (*Peviva Stockholm*) is a commercially available ELISA kit and experiments were carried out according to the manufacturer's instructions, as described previously (Cummings et al 2006). Plasma carcinoembryonic antigen (CEA) and lactate dehydrogenase (LDH) were also measured at these time-points as part of routine hospital standard of care blood testing.

Outcome measures

Standard follow up was carried out according to local clinical policy, with CT scans carried out approximately every 12 weeks. PFS duration was calculated from the date of consent to the date of disease progression as defined by RECIST or death (which ever occurred sooner). Patients who were alive at the end of the study had their PFS duration censored at the date of their last assessment.

Statistical Analysis

Significance testing was carried out using unpaired t-tests unless otherwise stated. Adjustments for multiple testing were not performed in this exploratory analysis. Kaplan-Meier Survival Curves were plotted using *Graphpad Prism* 6, and significance testing performed using log rank testing. For imaging parameter graphs, the range outside which results should be considered statistically significant was calculated using methods as described by Galbraith et al 2002. This method calculates the 95% confidence interval for a change which might occur in the patient cohort; any change in the measured parameter which is greater than this value is statistically significant at the 5% level (plotted as dotted lines on figures 1.2a, 1.2b, 1.4a, 1.5a and 1.5b).

RESULTS

Patient characteristics

Twenty patients with colorectal liver metastases underwent DCE-MRI and DW-MRI scanning with associated blood tests. The pre-treatment characteristics for the patients are listed in *Table 1.1*. The median age of the patients was 69.5 years, which is greater than most comparable trial populations. Mean pre-treatment CEA levels were high at 385µg/L; as was LDH at 2279 IU/L. The median PFS time was 8.7 months and median OS time was 17.3 months. All twenty patients underwent a minimum of one pre-treatment scan and one on-treatment scan and were considered as evaluable for response. No scan-related adverse events occurred. The reproducibility of MRI parameters was within expected limits with coefficients of variance of 8.0 % for median K^{trans} and 6.2 % for median ADC (Messiou et al 2012).

The effect of chemotherapy on measures of tumour vasculature

No pre-treatment MRI evaluation predicted response or duration of response (*figure 1.1*). The cohort K^{trans} was significantly higher on cycle 2 day 2 (C2D2) than before treatment and remained elevated at cycle 6 (*figure 1.2a*). There was a heterogeneous K^{trans} response to chemotherapy, with five patients having a significant increase in tumour median K^{trans} and five a significant decrease by C1D2 (*figure 1.2b*). This differential response was associated with survival. Patients with a tumour median K^{trans} higher than pre-treatment levels at C2D2 had an OS of 10.1 months whereas the equivalent statistic was 22.1 months in those without a significant rise at this time-point ($p = 0.049$, *figure 1.2c*).

The cohort iAUC was significantly increased on chemotherapy cycle 1 day 8 (C1D8) relative to pre-treatment and remained high at C2D2. It had reduced by cycle 6, but still remained significantly greater than pre-treatment readings. Again, a differential response was observed, with the tumours of six patients showing a significant decrease as early as C1D2 and six showing a significant increase by this time-point. This discord decreased by C1D8 and C2D2 but the tumour median iAUC readings were heterogeneous by cycle 6. These changes in iAUC were not of clinical significance in terms of outcome.

Cohort v_e increased significantly by C1D8 (average percentage change from pre-treatment 13.3%) and continued to rise on C2D2 (average 17.1%) and cycle 6 (average 34.9%). No

significant changes were seen in the cohort median tumour v_p readings. The cohort enhancing fraction was not altered significantly by chemotherapy.

CTCs were detectable in all twenty patients before commencing chemotherapy. The average number of CTCs was 5 CTCs per 7.5 ml blood (range 1-17 CTCs per 7.5 ml blood), with 11 patients categorized into the high CTC group (3 or more CTCs per 7.5 ml blood). A positive association existed between MRI-derived WTV and CTC number at the pre-treatment time-point (p = 0.005), *figure 1.3a.* A negative correlation was found between tumour median K^{trans} and CTC number before treatment (p = 0.042), *1.3b* and at C1D2 time-point (p= 0.018). Pre-treatment 'whole tumour permeability' was calculated (WTV x K^{trans}) to adjust for the bulk of disease. This measure also correlated significantly with CTC number (r = 0.5436, 95% CI 0.1185 to 0.8002, p = 0.016).

The plasma concentrations of the eighteen circulating angiogenesis-associated proteins lay within the dynamic range of the assay in all twenty patients. Chemotherapy led to detectable dynamic changes in IL8, VEGFR2, Ang1, FGFb, PDGFbb, VEGFA, VEGFC (down) and VEGFD and VCAM1 (up), *figure 1.4a.* Pre-treatment VEGFC and FGFb plasma concentrations correlated positively with PFS (VEGFC r= 0.56, p = 0.01; FGFb r = 0.50, p= 0.03; *figures 1.4b and 1.4c*)).

The effect of chemotherapy on tumour cell death and tumour volumes

The MRI-determined WTV fell significantly in all tumours by chemotherapy C2D2 and reduced further in all tumours by cycle 6 (C6). The mean percentage decrease in WTV was 29% (95% CI 18 to 42% decrease, p<0.0001) at C2D2 and 80% (95% CI 75 to 84% decrease, p<0.0001) at C6. There was no significant relationship between pre-treatment WTV or change in WTV and clinical outcome.

The median ADC in this cohort significantly increased by C2D2 (average percentage change 12.6%; 95% CI 4 to 21% increase, p = 0.004) and C6 (18.0%; 95% CI 6 to 30% increase, p = 0.005), *1.5a.* As with DCE-MRI and the circulating vascular markers, there was inter-patient heterogeneity in ADC response to chemotherapy (*1.5b*). Median OS was lower in patients with a significant rise in ADC at C2D2 but this did not reach statistical significance (11.6 months versus 24.0 months, p = 0.095), *figure 1.5c.*

Circulating levels of cytokeratin-18 (cCK18) were altered by chemotherapy. Most patients' plasma contained a lower concentration of cCK18 in comparison with pre-treatment levels but a distinct cohort showed an increase by cycle 6 (*figure 1.6a*). There was no significant difference in treatment outcome between these groups. cCK18 concentrations correlated positively with ADC on cycle one day eight (C1D8), $p = 0.034$, but not at any other time-points (*1.6b*).

DISCUSSION

This study examined the impact of chemotherapy on MRI and circulating markers of angiogenesis and apoptosis in twenty patients with colorectal cancer liver metastases. The patients in this cohort had a lower OS than was expected (17.3 months). This suggests they may have a poor prognosis as defined by; poor performance status, bulky disease with liver metastases of at least 3cm (required for clinical trial inclusion), high pre-treatment CEA and LDH levels and high pre-treatment CTC numbers.

These data demonstrate that oxaliplatin and 5FU or capecitabine chemotherapy regimens affect significantly the vascular MR parameters, in the form of rises in iAUC, K^{trans} and v_e. These increases were observed in some patients as early as 24 hours after the first dose of chemotherapy. Circulating angiogenesis related proteins were also affected by chemotherapy at early time-points and after 6 cycles of chemotherapy. These results imply that chemotherapy alone is exerting an effect on tumour vasculature, as previously hypothesised (Miller et al 2001). A rise in iAUC and K^{trans} may be a result of increased tumour capillary permeability caused by chemotherapy-induced capillary damage. Increased tumour perfusion can also contribute to a rise in these parameters and may be explained by cell death causing an increase in the extracellular extravascular volume, as measured by v_e, leading to decreased interstitial pressure and increased perfusion. A significant rise in K^{trans} by cycle 2 day 2 predicted for a poor overall survival (10.1 months) versus 22.1 months in those without this K^{trans} rise. This early predictor of response could potentially be of clinical use, to guide early treatment decisions. Bevacizumab has been shown to reduce K^{trans} in colorectal liver metastases (O'Connor et al 2011) and may be useful in this poor prognosis cohort of patients in whom K^{trans} rises with chemotherapy. This is a potential area for further study.

CTCs were detected in all patients, far more than the 61% previously described in this setting (Sastre et al 2008). The number of CTCs in the blood was proportional to the WTV before treatment commenced, implying that the number of cells released into the circulation is related to the tumour bulk (Cohen et al 2008). The numbers of CTCs also correlated with the K^{trans} of the tumours both at baseline and shortly after the first treatment was given (C1D2). As discussed above, this is consistent with the interpretation that chemotherapy increases tumour vessel permeability, accounting for the rise in K^{trans}, and leading to release of tumour cells into the circulation.

Chemotherapy led to detectable reductions in circulating VEGFA, VEGFC, VEGFR2, Ang1, FGFb, PDGFbb and IL8. These reductions in angiogenic and inflammatory markers are in keeping with the reduction of tumour volume detected following cytotoxic treatment and suggest an antiangiogenic effect of chemotherapy on the tumour vasculature. Circulating levels of VEGFD and VCAM1 were increased by chemotherapy treatment (these proteins were shed into the circulation as a result of treatment). VCAM-1 is a transmembrane glycoprotein, which is over-expressed on colorectal cancer cells and plays role in metastasis development and angiogenesis. Serum VCAM-1 is higher in patients with colorectal cancer than controls but serum levels do not appear to predict for response to treatment (Ciftci et al 2015). The effect of chemotherapy on serum levels has not been previously described in this setting. Pre-treatment levels of VEGFC and FGFb, which are central to regulation of tumour vasculature, positively correlated with duration of PFS.

The average WTV of the whole cohort of patients, as measured on MRI, was reduced from C1D8 onwards and this was sustained until at least cycle 6. This measure of WTV corresponded to treatment response (which is not surprising, given that the RECIST criteria are essentially measurements of tumour size) but did not predict duration of PFS or OS. Tumour cell death, as measured by WTV, occurs early in these tumours; a week following the first dose of chemotherapy. This is mirrored by a rise in ADC, a measure of cell death, as expected. OS duration was longer in patients with a significant rise in ADC at C2D2, although this did not reach statistical significance in this small cohort. This may prove to be a useful marker of response and warrants further investigation in a larger study.

Twelve out of twenty patients completed scanning protocols at chemotherapy cycle six, largely due to falling performance status by this time-point. This study was limited by the small numbers of participants and so should be regarded as exploratory rather than confirmatory.

In conclusion, imaging biomarkers and circulating biomarkers of angiogenesis and apoptosis were evaluated in patients with metastatic colorectal cancer treated with first line, oxaliplatin based chemotherapy. The imaging protocols were shown to be well tolerated and provided reproducible results. Significant early changes in MRI and circulating angiogenic biomarkers were observed following chemotherapy treatment alone, which must be taken into account when these chemotherapy regimens are used in

combination with molecularly targeted agents. A rise in K^{trans} by cycle 2 day 2 of chemotherapy correlated with shorter OS, which may be a useful early biomarker of lack of response. For the first time, a negative relationship between circulating tumour cells and K^{trans} has been demonstrated indicating a link between chemotherapy-induced changes in tumour vascular permeability and release of tumour cells into the circulation.

TABLES AND FIGURES

Characteristic	
Age (years)	
Median	69.5
Range	59-80
Sex (n)	
Male	17
Female	3
WHO performance status (n)	
0	7
1	12
2	1
Pre-treatment CEA (µg/L)	
Mean	384
Range	3- 2897
Pre-treatment LDH (IU/L)	
Mean	2279
Range	45 -11346
Chemotherapy regimen (n)	
Oxaliplatin and 5FU	18
Oxaliplatin and capecitabine	2
Previous adjuvant chemotherapy	
Yes	2
No	18

Table 1.1: Pre-treatment characteristics of study participants

	Post chemotherapy cycle 6 (number of patients)	Post chemotherapy cycle 12 (number of patients)
Complete response	0	1
Partial response	17	11
Stable disease	1	2
Progressive disease	2	4
Not applicable	0	2

Table 1.2: RECIST defined response to chemotherapy, as measured on CT scans.