Imaging Biomarkers of the Tumour Microenvironment to Assess Early Response in Patients Treated with Anti-Angiogenic Therapy

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Contents

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List of tables

List of Figures

Abstract

The University of Manchester
Laura Horsley
Doctor of Philosophy (PhD)
Imaging Biomarkers of the Tumour Microenvironment to Assess Early Response in Patients Treated with Anti-Angiogenic Therapy
2015

Background: Angiogenesis is the process by which new blood vessels develop from existing vasculature and is a critical step in all tumours to facilitate growth beyond a few millimetres. As this process is largely inactive in physiological circumstances in adults, it represents an attractive therapeutic target in oncology. Drugs that target the angiogenic process are classified as anti-angiogenic agents. The first anti-angiogenic drug to be approved by the FDA was bevacizumab; a recombinant humanized monoclonal antibody against VEGF. Randomised studies in colorectal cancer (and other solid malignancies) have reported prolonged progression free survival and overall survival for bevacizumab. However, standard radiological criteria, Response Evaluation Criteria In Solid Tumours (RECIST), although widely employed to assess response to therapy in clinical trials, are generally insensitive to the predominantly cytostatic effects of anti-angiogenic and other targeted therapies. Alternative methods of predicting or assessing early response to such agents are needed, particularly given the cost and toxicity implications of such treatments. However, biomarkers to aid selection of patients for anti-angiogenic therapies, including bevacizumab, remain elusive.

Purpose: To investigate Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI), Diffusion Weighted Imaging (DWI) and circulating angiocytokines, measured using an ELISA multiplex, as prognostic markers in patients with metastatic colorectal cancer treated with bevacizumab and chemotherapy.

Results: Seventy patients were treated. DCE-MRI and DWI parameters showed good reproducibility with coefficient of variation between 3.7 to 23% for parameters. The median progression free survival, the primary end point of the trial, was 9.3 months. The overall response rate was 44%. The clinical variables which were significant for progression free survival on univariate analysis were: performance status (p=0.005), CEA (p=0.04) and serum LDH (p=0.005). Biomarkers which were significant for progression free survival on univariate analysis were serum VEGF-A (p=0.02), serum HGF (p=0.005), sVEGFR-2 (p=0.02). In each case, low values of the biomarker were associated with improved outcome. Multivariate analysis identified K^{trans} (p=0.015), performance status (p=0.008) and serum HGF (p=0.003) as the most significant predictors of progression free survival. A prolonged progression free survival was associated with a good ECOG performance status, high K^{trans} and low serum HGF.

Conclusions: Whilst these results are encouraging, future work is required to establish whether HGF and K^{trans} are prognostic markers for metastatic colorectal cancer and their precise role in the prediction of patients likely to benefit from treatment with bevacizumab.

Declaration

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List of Abbreviations

Abbreviation	Definition
5-FU	5-Fluorouracil
ADC	Apparent Diffusion Coefficient
AE	Adverse Event
AIF	Arterial Input Function
Ang	Angiopoietin
B_0	Centre of MRI magnetic field
CAPOX	Capecitabine and Oxaliplatin
CEA	Carcinoembryonic Antigen
CoV	Coefficient of Variation
C_p	Tracer concentration in arterial blood plasma- input function
C_t	Concentration of contrast at time t in every voxel
CTCAE	Common Terminology Criteria for Adverse Events
CVA	Cerebrovascular Accident
DCE MRI	Dynamic Contrast Enhanced Magnetic Resonance Imaging
DWI-MRI	Diffusion-Weighted Imaging Magnetic Resonance Imaging
ECOG	The Eastern Cooperative Oncology Group
EES	Extracellular Extra Vascular Space
EF	Enhancing Fraction
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMEA	European Medicines Agency
EudraCT	European Clinical Trials Database
FA	Flip Angle
FDA	The US Food and Drug Administration Agency
FFE	Fast Field Echo
FGF	Fibroblast Growth Factor
FLT	18F fluorothymidine
FOLFIRI	Chemotherapy containing irinotecan and infusional 5FU
FOLFOX-4	Chemotherapy regimen containing oxaliplatin and 5FU
FOV	Field of View
GCP	Good Clinical Practice
GE	Gradient Echo
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HR	Hazard Ratio

HRE	Hypoxia Responsive Elements
IAUC	Initial Area Under the Curve
IFL	Chemotherapy regimen containing Irinotecan and bolus 5FU
IL	Interleukin
INR	International Normalized Ratio
IV	Intravenous
K_{ep}	Volume transfer constant EES→plasma
KGF	Keratinocyte Growth Factor
KRAS	Kirsten Ras
K^{trans}	Volume transfer constant plasma→EES
LDH	Lactate Dehydrogenase
mCRC	Metastatic Colorectal Cancer
MdG	Modified de Gramont
MVD	Microvessel Density
NCI	National Cancer Institute
NICE	National Institute for Clinical Excellence
NRP	Neuropilin
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSCLC	Non Small Cell Lung Cancer
OS	Overall Survival
PDGF	Platelet-Derived Growth Factor
PET	Positron Emission Technology
PFS	Progression Free Survival
PIGF	Placental Growth Factor
PS	Performance Status
RECIST	Response Evaluation In Solid Tumours
RF	Radiofrequency
ROI	Region Of Interest
T	Tesla
TE	Echo delay time
TIA	Transient Ischaemic Attack
TIC	Time Intensity Curve
TNM	Tumour Nodes Metastases tumour staging system
TR	Repetition time
V_e	Volume of EES/volume of tissue
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor

vHL	von Hippel Lindau
VOI	Volume Of Interest
V_p	Tumour blood volume/volume tissue

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Chapter 1: Introduction

1.1 Angiogenesis as a therapeutic target

Angiogenesis refers to the formation of new capillaries from pre-existing vessels. In healthy adults this process is active in only a few specific physiological conditions: endometrial growth during the menstrual cycle, embryonic growth, foetal growth and wound healing. Sustained angiogenesis is pathological and occurs in chronic inflammatory conditions such as rheumatoid arthritis and diabetic retinopathy, as well as playing an important role in the malignant growth of tumours and metastases. This has led to the description of angiogenesis as one of the hallmarks of cancer (Hanahan and Weinberg 2011) .

The concept that angiogenesis may be a rate limiting step in tumour growth, and thus a potential target for therapeutic intervention, was first put forward by Folkman (Folkman 1971). This emerged from the observation that to grow beyond a few mm^3, tumours need their own blood supply. The complex process of angiogenesis is tightly orchestrated by a large number of molecules some of which favour angiogenesis (pro-angiogenic) and others that inhibit angiogenesis (anti-angiogenic). The progression from quiescence towards angiogenic activity has been termed the “angiogenic switch” (Hanahan and Folkman 1996).

1.1.1 Activation of the angiogenic switch

A number of triggers leading to the angiogenic switch have been identified. These include environmental (epigenetic) factors such as hypoxia, acidosis and glucose deprivation. However, circulating factors such as cytokines (e.g. interleukin-6), steroid hormones, growth factors (e.g. basic fibroblast growth factor) and chemokines (e.g. stromal-cell-derived factor-1) are also important stimulants for the up regulation of angiogenesis.

Genetic factors such as mutations causing activation of oncogenes (e.g. Src and Ras) or inactivation of tumour suppressor genes (e.g. p53 and *vHL*) also result in the induction of angiogenesis.

Hypoxia is recognised as a particularly important stimulus to angiogenesis. A key mediator of the hypoxic response is hypoxia inducible factor-1 (HIF-1) and subsequent interaction between the von Hippel Lindau (*v*HL) tumour suppressor gene product (Hicklin and Ellis 2005). In the presence of normal oxygen tension, HIF-1α is broken down by the ubiquitin-proteosome pathway which is controlled by the *v*HL tumour suppressor gene product. In the hypoxic environment or, when *v*HL is mutated or absent, HIF-1α dimerizes with HIF-1β. The subsequent binding of the HIF-1α/HIF-1β complex to hypoxia responsive elements (HRE) on nuclear DNA results in the transcription of a number of genes important in the regulation of angiogenesis, including vascular endothelial growth factor (VEGF).

Twenty-seven endogenous negative regulators of angiogenesis have been isolated. These are shown in Table 1.

Inhibitor	Mechanism	Reference
Angiostatin	Inhibits endothelial cell proliferation and migration	(O'Reilly et al, 1994)
Arresten	Inhibits endothelial cell tube formation, proliferation and migration	(Colorado et al, 2000)
Anti-thrombin III	Inhibits endothelial cell proliferation	(O'Reilly et al, 1999)
Canstatin	Inhibits endothelial cell tube formation and migration	(Kamphaus et al, 2000)
Chondromodulin	Inhibition of endochondral ossification	(Kusufuka et al, 2002)
Collagen fragments	Inhibition of endothelial cell proliferation and migration in vitro. Inhibition of tumor-growth in vivo.	(Marneros and Olsen 2001)
EFC-XV	inhibits migration of endothelial cells	(Nyberg, Xie et al. 2005)
Endorepellin	Inhibit tumour neovascularization and tumour growth in vivo	(Mongiat et al, 2003)
Endostatin	Inhibition of endothelial cell migration and survival	(Abdollahi, et al, 2004)
Fibronectin fragment (Anastellin)	Inhibits tumour growth, angiogenesis and metastasis in pre-clinical models	(Yi and Ruoslahti 2001)
Fibulin	Inhibition of endothelial cell proliferation and invasion	(Albig et al, 2004)
FKBPL	Inhibition of endothelial cell and tumour cell migration via CD44 mediated signalling	(Valentine, O'Rourke et al. 2011)
Interferons	Inhibits endothelial cell proliferation and migration. Inhibition of IL-8 and down regulation of bFGF and VEGF gene expression	(Von Marshall et al, 2003)
Interleukins(IL-4 and-12)	IL-4 inhibits bFGF ;IL-12 stimulates IFN-γ production	(Volpert et al, 1998) (Voest et al, 1995)

Table 1: Endogenous inhibitors of angiogenesis

Table continues overleaf

Inhibitor	Mechanism	Reference
2-Methoxyestradiol	induces tumour and endothelial apoptosis	(Pribluda et al, 2000)
Pigment epithelial derived factor (PEDF)	Inhibits vasculogenesis	(Abe et al, 2004)
PEX	Inhibition of endothelial cell invasion	(Brooks et al, 1998)
Plasminogen Kringle 5	Inhibits endothelial cell migration and proliferation	(Cai, Zhang et al. 2012)
Platelet factor-4	Inhibits FGF-2 mediated endothelial cell proliferation	(Maione et al, 1990)
Prolactin fragments	Inhibition of VEGF stimulated MAPK signalling leads to reduced endothelial cell proliferation	(D'Angelo et al, 1995)
Prothrombin Kringle 2	Inhibits endothelial cell proliferation	(Lee et al, 1998)
sVEGFR-1	Sequestration of VEGF	(Belgore et al, 2000)
Thrombospondin -1 and -2	Inhibition of endothelial cell tube formation, migration and induces endothelial apoptosis	(Noh et al, 2003)
TIMPS	Inhibit endothelial cell proliferation	(Seo et al, 2003)
Troponin-1	Inhibits endothelial cell proliferation	(Feldman et al, 2002)
Tumstatin	Inhibits endothelial cell proliferation and promotes endothelial cell apoptosis	(Maeshima et al,2002)
Vasostatin	Inhibits endothelial cell proliferation and tumour growth in pre-clinical models	(Yao et al, 2002)

Table 1 continued: Endogenous inhibitors of angiogenesis

1.1.2 Mediators of angiogenesis

The major mediators of both normal and pathogenic angiogenesis are the vascular endothelial growth factor (VEGF) family of proteins and receptors. The activation of the VEGF-receptor (VEGFR) via VEGF, initiates a cascade of signalling pathways resulting in the up-regulation of genes that are involved in endothelial cell survival, differentiation, migration, proliferation, vessel permeability and the mobilization of endothelial progenitor cells from the bone marrow into the circulation (Dvorak 2002).

1.1.3 VEGF and its receptors

The VEGF family of growth factors consists of six structurally related proteins: VEGF-A (prototypic VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factors (PlGF1 and 2). First described by Dvorak as a vascular permeability factor secreted by tumour cells (Senger, Galli et al. 1983), VEGF- A is a 34-45-kd protein with a number of different angiogenic properties which is over-expressed in a number of solid tumours(Ferrara and Alitalo 1999). Preclinical studies have shown that VEGF-A is critical for normal development. Deletion of one or both alleles of the VEGF-A gene, located on the short arm of chromosome 6, results in vascular and cardiac abnormalities lethal to the developing embryo.

Twelve isoforms of VEGF have been demonstrated through alternative exon splicing of the VEGF gene (Nowak, Woolard et al. 2008), of which $VEGF_{165}$ is the predominant isoform. The smaller VEGF isoforms are freely soluble, whereas the larger proteins are bound to the extra-cellular matrix (ECM) and require protease activation. Most of these isoforms activate signal transducing receptors. However, inhibitory isoforms have also been described and designated as VEGF-Axxxb; where xxx relates to the number of amino acids in the protein.

A pre-clinical study using colorectal cancer cell lines demonstrated that response to bevacizumab was dependant on the balance between pro-angiogenic and anti-angiogenic VEGF isoforms (Varey, Rennel et al. 2008). However, whether this is relevant in humans, remains to be proven.

Although most VEGF-A is expressed by tumour cells, expression has been found in vascular endothelial and stromal cells, particularly in hypoxic areas (Fukumura, Xavier et al. 1998).

The function of VEGF-B has remained largely unknown although recently, pre-clinical researchers have proposed a critical role for VEGF-B in the survival of formed blood vessels in pathological conditions (Zhang, Tang et al. 2009). Both VEGF-C and VEGF-D are important for embryonic and post natal lymphangiogenesis and a role in the growth of new blood vessels in tumours has also been speculated (Hicklin and Ellis 2005). VEGF-E is a viral homolog of VEGF-A, produced by the parapoxvirus Orf virus.

Three high affinity VEGF tyrosine-kinase receptors have been identified: VEGF receptor (VEGFR)-1 (also known as fms-like tyrosine kinase or Flt-1), VEGFR-2 (also called KDR) and VEGFR-3 (also known as fms-like tyrosine kinase 4 or Flt-4). The various members of the VEGF family have different binding affinities for each of these receptors which are variably expressed on the surface of endothelial cells and in the case of VEGFR-1 and -2; adult haematopoietic cell lines.

Although VEGF binds to VEGFR-1 with high affinity, the resultant signal transduction is biologically less significant than that induced by VEGFR-2. Vascular endothelial growth factor-B and PlGF uniquely bind to VEGFR-1. Preclinical evidence from VEGFR-1 deficient mice indicates a critical role for the receptor in developmental angiogenesis. A soluble form of VEGFR-1 (sVEGFR-1) has been identified in the serum of pregnant women and pathologically in some tumours and is thought to reduce or modify the action of VEGF.

Receptor-ligand binding between VEGF and VEGFR-2 has a critical role in angiogenesis via tyrosine kinase phosphorylation and activation of the Raf-Mek-Erk pathway leading to microvascular permeability as well as endothelial proliferation, survival, migration and invasion.

Neuropilins -1(NRP-1) and -2 (NRP-2) serve as co-receptors for VEGF by increasing binding affinity for ligands to receptors. Unlike VEGFR, these receptors lack an intra-cellular signalling domain. Results from recent studies suggest that NRP receptors may act independently of VEGFR (Wang, Dutta et al. 2007), whereas previously they were thought to act purely as modulators for VEGFR.

1.1.4 High levels of VEGF expression correlate with clinical outcome

Studies in a variety of malignancies including colorectal cancer (Takahashi, Kitadai et al. 1995; Lee, Chow et al. 2000), breast cancer (Gasparini and Harris 1995), ovarian cancer (Shen, Ghazizadeh et al. 2000), renal cancer (Jacobsen, Grankvist et al. 2004) and lung cancer (Fontanini, Lucchi et al. 1997) indicate that high levels of VEGF expression correlate with adverse prognostic factors such as tumour size, the development of metastases and survival. In colorectal cancer, increased VEGF expression occurs in both the primary tumour and related metastases (Lee, Chow et al. 2000).

The assessment of circulating VEGF in vivo from blood samples is convenient. A number of studies in different cancers have demonstrated that circulating VEGF is associated with a heavy disease burden and extent of metastases (Salven, Manpaa et al. 1997; Fuhrmann-Benzakein, Ma et al. 2000). Other researchers (Davies, Jonas et al. 2000) found that plasma VEGF levels in colorectal cancer correlated with the volume of liver metastases as measured by computerised tomography. Different methods for assessing VEGF and the role of VEGF as a potential biomarker for response to anti-angiogenic therapy will be discussed later in the introduction.

1.1.5 The development of anti-angiogenic strategies

The importance of angiogenesis in tumour development has led to widespread research in the development of targeted therapies aimed at various aspect of the angiogenic pathway. Drugs targeting this pathway are described as “anti-angiogenic”.

Currently, there are over a thousand trials registered with the National Cancer Institute (NCI) involving anti-angiogenic agents; over a hundred of which involve new agents. At the time of writing there are eleven anti-angiogenic drugs which are approved by the FDA for use in solid tumours, summarised in Table 2. Bevacizumab, a humanised monoclonal antibody, was the first anti-angiogenic based therapy to be approved by the FDA

1.1.6 Bevacizumab

Approval for the use of bevacizumab was first granted by the FDA in 2004 based on the results from a pivotal phase III trial in metastatic colorectal cancer. This trial in previously untreated patients compared the combination of chemotherapy drugs irinotecan and 5FU, with the addition of bevacizumab or a placebo (Hurwitz, Fehrenbacher et al. 2004). Results showed a dramatic difference in favour of the bevacizumab containing arm for progression free survival (PFS), 10.6 v 6.2 months ($p<0.001$), and overall survival (OS) 20.3 v 15.6 months ($p<0.001$). Subsequently, bevacizumab has been used widely in the treatment of metastatic colorectal cancer particularly in the United States of America (USA) and mainland Europe, where use was approved by the European Medicines Authority (EMA). However, an unfavourable cost benefit analysis from a health technology assessment undertaken in England and Wales (Tappenden, Jones et al. 2007), meant that bevacizumab was not approved for use by the National Institute for Clinical Excellence (NICE) for the treatment of metastatic colorectal cancer.

Drug	Class	Molecular target	FDA approved indication
bevacizumab	monoclonal antibody	VEGF-A	colorectal cancer glioblastoma non-small cell lung cancer renal cell cancer
ramicirumab	monoclonal antibody	VEGFR-2	gastric cancer
aflibercept	soluble decoy receptor	VEGF A and B PlGF	colorectal cancer
axitinib	tyrosine kinase inhibitor	(VEGFR)-1,-2 and -3	renal cell cancer
pazopanib	tyrosine kinase inhibitor	(VEGFR)-1,-2 and -3, (PDGFR)-α and –β. Kit.	renal cell cancer soft tissue sarcomas
regorafenib	tyrosine kinase inhibitor	(VEGFR)-1, -2, -3, PDGFR, KIT, RET, RAF, BRAF and BRAF V600E. FGFR, TIE-2,	colorectal cancer
sorafenib	tyrosine kinase inhibitor	(VEGFR),-2 and -3, (PDGFR)-β CRAF	hepatocellular carcinoma renal cell cancer
sunitinib	tyrosine kinase inhibitor	(VEGFR)-1,-2 and -3. (PDGFR)-α and –β. Kit FLT3 CSF-1R RET	GI stromal tumours pancreatic neuroendocrine tumours renal cell cancer
vandetanib	tyrosine kinase inhibitor	VEGFR EGFR	Medullary cell thyroid cancer
everolimus	mTOR pathway inhibitor	mTOR pathway	renal cell cancer pancreatic neuroendocrine cancer
temsirolimus	mTOR pathway inhibitor	mTOR pathway	renal cell cancer non-hodgkins lymphoma

Table 2: Anti-angiogenic drugs approved by the FDA for use in solid tumours

Trials in other cancers have also shown clinical benefit with the addition of bevacizumab to chemotherapy. Phase II trials in renal cancer (Yang, Haworth et al. 2003) and glioblastoma (Vredenburgh, Desjardins et al. 2007) have shown improvements in PFS with bevacizumab treatment leading to FDA approval for bevacizumab use in these disease sites.

In breast cancer, a phase III trial (Miller, Wang et al. 2007) demonstrated that the addition of bevacizumab to chemotherapy with paclitaxel resulted in an improvement in the median PFS from 5.9 months to 11.8 months (HR 0.6, $p<0.001$). Although the FDA approved the use of bevacizumab in breast cancer based on these data, approval was later rescinded following a lack of benefit in overall survival and safety concerns regarding an increased risk of cardiac toxicity in this population. In non -small cell lung cancer (Sandler, Gray et al. 2006), an improvement in PFS from 4.5 to 6.2 months and an overall survival benefit of 2.3 months in compared with chemotherapy alone, led to FDA approval. However NICE felt that this was insufficient to offset the increased toxicity. More recently trials in advanced ovarian cancer (Perren, Swart et al. 2011) combining chemotherapy and bevacizumab have demonstrated improvements in PFS and OS, leading to FDA and EMEA approval for bevacizumab in ovarian cancer.

It is clear from these trials that as overall improvements in PFS and OS are modest there is a need to define who benefits from the addition of bevacizumab in order to reduce toxicity and cost. A method of selecting patients likely to benefit from the addition of bevacizumab to standard therapy is being pursued by researchers and would be a major breakthrough in the treatment of patients with solid tumours.

1.1.7 Vessel normalization

A theory of vessel normalization and remodelling has been proposed as an explanation of why treatment with bevacizumab appears to act synergistically with chemotherapy (Carmeliet and Jain 2011).

Evidence to support this hypothesis has come from a number of clinical studies investigating VEGF pathway inhibitors ((Willett, Boucher et al. 2004; Batchelor, Sorensen et al. 2007). These studies demonstrated a period following treatment called "a normalization window" where reduced endothelial proliferation, decreased vessel density and permeability led to increased oxygenation and perfusion. In vivo, this has been most effectively demonstrated by a reduction in vessel permeability estimated by the Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) parameter K^{trans} (Willett, Boucher et al. 2005; Batchelor, Sorensen et al. 2007). The investigation of response to bevacizumab using DCE-MRI is an important part of this thesis and is discussed in more detail later in this introduction.

1.2 Colorectal cancer

1.2.1 Epidemiology

Globally, colorectal cancer is the third most common cause of cancer and the fourth most common cause of cancer death (Brenner, Kloor et al. 2013). In the UK, colorectal cancer is the second major cause of cancer death, accounting for 16,000 deaths per year (CRUK website). This high mortality is partly because many patients present late with advanced disease. Recognition of this fuelled the development of a national bowel screening project which was established in 2006 and, in England, offers two yearly screening to all men and women aged 60-69. In England, the programme is gradually being extended to include 70-74year olds, in line with other parts of the United Kingdom and sigmoidoscopy for adults aged 55 years, is expected to be added to the existing screening programme from 2015. This follows results from a large randomised trial which showed a 40% reduction in mortality at 11years of follow after a single sigmoidoscopy in 55-64 year olds (Atkin, Edwards et al. 2010).

1.2.2 Risk Factors

The incidence of colorectal cancer increases with age. However, a family history of colorectal cancer, particularly affecting first degree relatives and a personal history of inflammatory bowel disease was found to double the risk of colorectal cancer in population studies. Around 5-10% of colorectal cancers are due to the genetic conditions: Hereditary Non-Polyposis Colorectal Cancer (HNPCC or Lynch Syndrome) and Familial Adenomatous Polyposis (FAP). Environmental risk factors for colorectal cancer include: diet, inactivity, smoking and alcohol consumption.

1.2.3 Staging

Cancers may arise from any part of the large bowel but are most commonly found on the left side of the bowel in the sigmoid colon and rectum.

Adenocarcinoma is the most common histological sub-type of colorectal cancer and in most cases arises following malignant transformation of a dysplastic adenomatous polyp. Both the treatment and prognosis of colorectal cancer depend on the stage at diagnosis, which is classified according to the TNM staging system: with T representing tumour depth of invasion, N representing nodal involvement and M for metastatic involvement.

1.2.4 Treatment of advanced colorectal cancer

Advanced disease is the term given to patients with evidence of distant metastases, the presence of which are typically assessed by radiological staging.

The liver is the most common site of distant metastatic spread in colorectal cancer with 50% of patients overall developing liver metastases. Around 15-20% of patients with liver only metastatic disease are suitable for liver resection, with some specialist centres reporting 10 year survival rates of 25% (Rees, Tekkis et al. 2008). Historically, patients with disease which was not immediately suitable for potentially curative surgery were treated with palliative intent.

However improvements in response rates from combination chemotherapy and better surgical techniques, have led to approximately 10-15% of patients in this latter “unresectable” group becoming suitable for liver resection following a response to chemotherapy (Adam, Wicherts et al. 2009).

For many years single agent chemotherapy with Fluorouracil (5-FU) was the international standard of care for the treatment of advanced colorectal cancer. Historically, the median survival with 5-FU alone was around 10 months (Petrelli, Herrera et al. 1987). A small randomised trial found this was superior to best supportive care alone by approximately 5 months (Scheithauer, Rosen et al. 1993).

The introduction of new chemotherapy drugs called irinotecan and oxaliplatin represented a further major breakthrough in treatment for metastatic colorectal cancer. A randomized trial comparing the combination of irinotecan and 5-FU with 5-FU alone established a clear overall survival benefit of 17.4 months versus 14.1 months ($p=0.031$) in favour of the irinotecan containing arm (Douillard, Cunningham et al. 2000). The introduction of oxaliplatin combined with 5FU in a modified de Gramont (MdG) regimen also improved median survival compared with 5FU alone (de Gramont, Figer et al. 2000). Thus combination treatment with 5FU and oxaliplatin or irinotecan, irrespective of the order of sequencing (Tournigand, Andre et al. 2004), quickly became established as the standard of care for metastatic colorectal cancer and led to a significantly improved median overall survival of 22 months.

1.2.5 Prognostic factors in the treatment of advanced colorectal cancer

The outcome following chemotherapy in advanced colorectal cancer is influenced by a number of factors. One of the most powerful pre-treatment predictors of outcome is the WHO performance status (PS) (Sargent, Kohne et al. 2009). Patients who have a PS of 0 or 1, have a much better survival in multivariate analyses than those with PS of 2 or more (Kohne, Cunningham et al. 2002; Sorbye, Kohne et al. 2007; Chibaudel, Bonnetain et al. 2011).

Other important factors include elevation of white blood cell (WBC) count; elevation of alkaline phosphatase (ALP) ≥300U/l and the number of metastatic sites. The presence of liver or peritoneal metastases; haemoglobin <11.0 x 10^9/l and platelets ≥400 x 10^9/l have also been shown to adversely affect prognosis in some studies (Kohne, Cunningham et al. 2002).

Elevation of serum lactate dehydrogenase (LDH) has recently been identified as an independent prognostic variable (Chibaudel, Bonnetain et al. 2011) and has been proposed as a biomarker for highly angiogenic tumours (Scartozzi, Giampieri et al. 2012). Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion which has been validated as a biomarker for monitoring response to treatment in colorectal cancer (Locker, Hamilton et al. 2006). There is evidence that high pre-operative levels of CEA are an adverse prognostic factor in colorectal cancer (Andicoechea, Vizoso et al. 1998)

1.2.6 Targeted therapies in advanced colorectal cancer

Subsequent strategies to improve survival in colorectal cancer have focused on the addition of mechanism based targeted therapies to chemotherapy. The major classes of targeted therapy, which have been tested in clinical trials involving patients with metastatic colorectal cancer, have been those involving the down regulation of epidermal growth factor receptor (EGFR) pathway or targeting the angiogenesis pathway. These mechanism based treatments have limited single agent activity and are largely used in combination with cytotoxic chemotherapy drugs. Financial considerations and concerns regarding toxicity have driven the search to identify predictive biomarkers in order to identify the patients most likely to respond to these treatments.

Cetuximab is one of two monoclonal antibodies targeting the EGFR receptor which are licensed for the treatment of metastatic colorectal cancer.

Retrospective analyses of two pivotal randomised phase III trials demonstrated that treatment benefit from the addition of Cetuximab to chemotherapy, was limited to a sub-group of patients without mutations of the KRAS gene (KRAS wild type), (Karapetis, Khambata-Ford et al. 2008; Van Cutsem, Kohne et al. 2011). The 30-40% of colorectal cancer tumours that show evidence of KRAS mutations are resistant to antibody directed EGFR inhibition due to constitutive activation of the KRAS protein.

As cetuximab and other drugs in this class are expensive and have unpleasant side effects, the EMEA restricted the use of these antibodies to patients without a mutation in exon 2 of the KRAS gene.

Recent publications from further trials have identified mutations in KRAS exons 3 or 4 or NRAS exons 2-4 that confer reduced benefit to EGFR antibody therapy. The PRIME study evaluated the combination of Panitumumab and FOLFOX-4 chemotherapy versus chemotherapy alone in 1183 patients with metastatic colorectal cancer according to KRAS exon 2 mutation statuses. The prevalence of mutations occurring in exons 3 or 4 of KRAS or exons 2-4 NRAS was 17% in patients with wild type exon 2 KRAS (Douillard, Oliner et al. 2013). A planned subgroup analysis established that the absence of any RAS mutations was associated with a longer progression free survival in the experimental arm: 10.1months versus 7.9 months (HR 0.72, 95% CI 0.58-0.90; p=0.004). There was also a 6 month improvement in overall survival in favour of the experimental arm for this subgroup (HR 0.72, 95% CI 0.58-0.90; p=0.04). The prevalence of BRAF exon 15 mutations was 10% in the PRIME trial. Patients with this mutation had a poorer outcome in terms of PFS and OS in both treatment arms, consistent with previous observations of BRAF V600E mutation as a prognostic biomarker in metastatic colorectal cancer (Farina-Sarasqueta, van Lijnschoten et al. 2010; Van Cutsem, Kohne et al. 2011).

The FIRE-3 study (Heinemann, von Weikersthal et al. 2014) compared the efficacy of FOLFIRI plus bevacizumab versus FOLFIRI plus cetuximab in the first line treatment of 592 patients with KRAS exon 2 wild-type metastatic colorectal cancer. Although there was no significant difference in response rates or progression free survival between allocated treatments, there was a difference in overall survival in favour of the FOLFIRI plus Cetuximab combination: 28.7 months versus 25 months (HR 0.77, 95% CI 0.62-0.96; p=0.017). Furthermore, a planned retrospective analysis of the subgroup of patients who were wild type at all tested loci, (including exons 3 or 4 of KRAS or exons 2-4 NRAS) indicated that the survival advantage of the cetuximab combination was even greater: 33.1 months versus 25.6 months (HR 0.70, 95% CI 0.53-0.92; p=0.011). The results from the PEAK study which compared 278 patients treated with either, FOLFOX-6 and panitumumab or FOLFOX-6 and bevacizumab have corroborated these findings (Schwartzberg, Rivera et al. 2014).

As a consequence of these trial results it has now become standard of care to carry out mutation testing for exons 2,3 and 4 of KRAS and exons 2,3 and 4 NRAS, as it would appear that each of these RAS mutations are negative predictive biomarkers for EGFR antibody therapy.

1.3 Phase II and III trials of anti-angiogenic agents in colorectal cancer

The seminal trial (Hurwitz, Fehrenbacher et al. 2004), which compared bevacizumab or placebo in addition to irinotecan and 5FU chemotherapy, showed a significant increase in progression free survival (PFS) from 6.2 to 10.6 months. Overall survival (OS) was also significantly improved from 15.6 to 20.3 months in favour of the bevacizumab combination arm. Since then, a large randomised phase III trial (Saltz, Clarke et al. 2008), investigating oxaliplatin and 5FU chemotherapy with bevacizumab or placebo, failed to replicate such a significant survival advantage and showed only a modest benefit of 1.4 months in terms of progression free survival.

The discordance in results between these two large studies (Hurwitz, Fehrenbacher et al. 2004; Saltz, Clarke et al. 2008) has been attributed to the high treatment discontinuation rate in the NO16966 trial (Saltz, Clarke et al. 2008). In contrast, patients in the Hurwitz study could continue bevacizumab beyond progression, with second line therapy. This may also explain the negative findings in the smaller TREE study (Hochster, Hart et al. 2008), which used oxaliplatin and 5FU chemotherapy, and had high treatment discontinuation rates of 47-51% depending on treatment arm. This suggests that treatment until progression could be important in maximising both the effect of bevacizumab and potentially other anti-angiogenic drugs.

Subsequently, there have been several other trials combining anti-angiogenic VEGF inhibitors with chemotherapy in metastatic colorectal cancer. Results of published phase II and III trials of anti-angiogenic agents in the first line treatment of metastatic colorectal cancer are shown in Tables 3-5.

Type of study	N	Treatment	PFS (months)	OS(months)	Reference
Randomised phase II	104	Rosewell park (RP) RP + B 5mg/kg RP + B 10mg/kg	5.2 9 (p=0.005) 7.2	13.8 21.5 NS 16.1	(Kabbinavar, Hurwitz et al. 2003)
Randomised phase II	209	Rosewell park (RP) RP + B 5mg/kg	5.5 9.2 (p= 0.0002)	12.9 16.6 NS	(Kabbinavar, Schulz et al. 2005)
Open label phase III (AVEX)	280	capecitabine (cap) cap +B 7.5mg/kg	5.1 9.1 (p<0.0001)	Not reported	(Cunningham, Lang et al. 2013)

Table 3: Trials in metastatic colorectal cancer of fluoropyrimidines in combination with bevacizumab

B = Bevacuzimab, NS = not significant

Type /name of study	N	Treatment	PFS (months)	p	OS(months)	p	Reference
Randomised phase III (AVF2107)	813	IFL + placebo IFL +B 5mg/kg	6.2 10.6	<0.001	15.6 20.3	<0.001	(Hurwitz, Fehrenbacher et al. 2004)
Single arm phase II (E2200)	81	IFL +B 10mg/kg	10.7		26.3		(Giantonio, Levy et al. 2006)
Randomised phase III 2nd line (E3200)	829	FOLFOX4 alone FOLFOX + B 10mg/kg B 10mg/kg	4.7 7.3 2.7	<0.001	10.8 12.9 10.2	0.0011	(Giantonio, Catalano et al. 2007)
Randomised phase II (BICC-C)	117	FOLFIRI+B 5mg/kg IFL + B 5mg/kg	11.2 8.3	NS	28 19.2	0.037	(Fuchs, Marshall et al. 2007)
Randomised phase III (NO16966)	1401	XELOX /FOLFOX-4 + placebo XELOX/FOLFOX-4 + B 2.5mg/kg/week	8 9.4	0.0023	19.9 21.3	NS	(Saltz, Clarke et al. 2008)
Randomised phase III (TREE)	223	mFOLFOX-6+ B 5mg/kg bFol +B 5mg/kg CapOx + B 7.5mg/kg	9.9 8.3 10.3		26.1 20.4 24.6		(Hochster, Hart et al. 2008)
Single arm phase IV (AVIRI)	209	FOLFIRI + B 5mg/kg	11.1		22.2		(Sobrero, Ackland et al. 2009)
Single arm phase II	43	FOLFIRI + B 5mg/kg	12.8		31.3		(Kopetz, Hoff et al. 2010)
Randomised phase III (MAX)	471	Capecitabine Capecitabine +B 7.5mg/kg Cap +M+B 7.5mg/kg	5.7 8.5 8.4	<0.001	18.9 18.9 16.4	NS	(Tebbutt, Wilson et al. 2010)

Table 4: Trials in metastatic colorectal cancer of combination chemotherapy with bevacizumab

B = bevacizumab, NS = not significant

Type / name of study	N	Treatment	PFS (months) p	OS(months) p	Reference
Randomised phase III (CONFIRM-1)	1168	FOLFOX4 + placebo FOLFOX4 + vatalanib	7.7 NS 7.6	20.5 NS 21.4	(Hecht, Trarbach et al. 2011)
Randomised phase III (HORIZON II)	860	FOLFOX/CAPOX + placebo FOLFOX/CAPOX +cediranib	8.3 p=0.121 8.6	18.9 NS 19.7	(Hoff, Hochhaus et al. 2012)
Randomised phase III (HORIZON III)	1422	mFolfox6 +bevacizumab mFolfox6 +cediranib	10.3 NS 9.9	21.3 NS 22.8	(Schmoll, Cunningham et al. 2012)
Randomised phase III (SUN 1122)	768	FOLFIRI +placebo FOLFIRI+ sunitinib	8.4 7.8 NS	Not reported	(Carrato, Swieboda-Sadlej et al. 2013)
Randomised phase IIB (RESPECT)	198	mFolfox6+placebo mFolfox6+sorafenib	8.7 9.1 NS	18.1 17.6 NS	(Tabernero, Garcia-Carbonero et al. 2013)
Randomised phase II	126	mFOLFOX6 + axitinib mFOLFOX6 + B mFOLFOX6 + axitinib +B	11 15.9 12.5	18.1 21.6 19.7	(Infante, Reid et al. 2013)

Table 5: Important trials in metastatic colorectal cancer of combination chemotherapy and tyrosine kinase inhibitors

A recent pooled analysis of 7 randomised controlled trials containing bevacizumab in metastatic colorectal cancer involving 3763 patients (Hurwitz, Tebbutt et al. 2013) has demonstrated an overall survival benefit in favour of bevacizumab (HR 0.80, 95% CI, 0.71-0.90, p=0.0003). A significant improvement in PFS across trials was also confirmed (HR 0.57, 95% CI 0.46-0.71, p<0.0001).

1.3.1 Duration of bevacizumab therapy

The first phase II trial involving bevacizumab in colorectal cancer (Kabbinavar, Hurwitz et al. 2003) involved the administration of the antibody until disease progression or 48 weeks; whichever occurred first, with the option of crossover to bevacizumab on progression in the control group. In the experimental arm, if disease progressed within 6 months, bevacizumab could be restarted in an open label extension study. In the pivotal AVF2107 trial, patients could discontinue chemotherapy in the event of unacceptable side effects and continue bevacizumab alone. The N016966 trial undertaken by Saltz and colleagues, also allowed individual drugs to be discontinued and continuation of the remaining drug(s) until toxicity or progression. However, many physicians failed to implement this resulting in premature discontinuation of therapy for many patients. The variable approach to maintenance therapy is an important difference between these two trials which may explain the difference in PFS.

Maintenance bevacizumab following induction chemotherapy for patients with metastatic colorectal cancer has recently been tested by a Spanish group who conducted a non-inferiority study investigating bevacizumab alone or combined with chemotherapy following induction chemotherapy (Diaz-Rubio, Gomez-Espana et al. 2012). The difference in PFS and OS between the treatment arms was 0.7 and 2.8 months respectively suggesting a possible role for maintenance bevacizumab alone. However the trial was insufficiently powered to meet the non-inferiority end-point.

These findings are consistent with recent reports from a Swiss group of researchers who conducted an open label phase 3 non-inferiority study in 262 patients with stable disease 4-6 months following first line treatment (Koeberle, Betticher et al. 2015) . Patients were randomised to maintenance bevacizumab or observation. At a median follow up of 36.7 months, non inferiority was not demonstrated in the observation arm.

More encouraging results were reported by the CAIRO-3 study investigators who randomised 558 patients with stable disease, following 6 cycles of first line therapy with capecitabine, oxaliplatin and bevacizumab (CAPOX-B), to observation or maintenance treatment with capecitabine and bevacizumab (Koopman, Lieke HJ Simkens et al. 2013). Both groups of patients were re-treated with CAPOX-B at the time of first progression, which was termed PFS1. Maintenance treatment significantly improved PFS1, 4.1 months vs 7.4 months (HR 0.44, 95% CI 0.37-0.54, $p<0.0001$). There was also a significant improvement in overall survival in favour of the patients who received maintenance therapy 17.9 months vs 21.7 months (HR 0.77, 95% CI 0.62-0.96, $p<0.02$), although only 44% of patients in the maintenance arm received re-treatment with CAPOX-B following first progression compared with 72% in the observational group. However, a limitation of this study was the absence of a comparator arm with capecitabine alone.

Since endothelial cells are genetically stable compared to cancer cells, it has been hypothesized that continuation of anti-angiogenic therapy could be beneficial when switching chemotherapy at disease progression. Resistance to anti-angiogenic therapies is thought to occur due to the development of alternative anti-angiogenic pathways and there is no evidence this occurs simultaneously with biological resistance to chemotherapy. An observational cohort study involving 1953 patients (Grothey, Sugrue et al. 2008) showed a significant survival advantage in 642 patients who continued bevacizumab with second line therapy compared with 531 patients receiving chemotherapy alone (38.1 months v 19.9 months).

A benefit for bevacizumab beyond progression has recently been confirmed in a prospective phase III clinical trial carried out by European investigators who randomised 820 patients between continuation of bevacizumab and chemotherapy alone at progression (Bennouna, Sastre et al. 2013). Results showed a 1.6 month difference in PFS between the treatment arms and an improvement in overall survival from initiation of second line therapy from 9.8 months to 11.2 months in favour of the arm continuing bevacizumab (HR 0.81, p=0.0062). Other recent studies in colorectal cancer have also demonstrated activity of anti-angiogenic therapies aflibercept and regerafenib, in patients previously treated with bevacizumab (Van Cutsem, Tabernero et al. 2012; Grothey, Cutsem et al. 2013). Taken together these results provide evidence of a role for anti-angiogenic therapy beyond first line chemotherapy.

1.3.2 Dose level of bevacizumab

The original phase one trial of bevacizumab (Gordon, Margolin et al. 2001) investigated an intra-venous dose of 0.1mg/kg to 10mg/kg, with no evidence of dose limiting toxicity at higher dose levels. Pharmacokinetic studies showed a plasma half-life of 21 days and a linear relationship between dose level and plasma concentrations of drug. Doses of ≥ 0.3.mg/kg resulted in free plasma VEGF concentration below the detectable limit of the assay. A later phase one trial combining bevacizumab and chemotherapy used a dose of 3mg/kg (Margolin, Gordon et al. 2001).

Although a dose level of 2.5mg/kg/week of bevacizumab has been commonly used in colorectal cancer, there are conflicting data as to whether this dose is optimum. Several trials have investigated different dose levels of bevacizumab in mCRC. A small phase 1 trial combining bevacizumab with chemo-radiotherapy (Willett, Boucher et al. 2005) showed a higher pathological response rate for patients treated with 5mg/kg/week rather than 2.5mg/kg/week. However, a small phase II trial (Kabbinavar, Hurwitz et al. 2003) that compared 5mg/kg/week and 2.5mg/kg/week reported lower response rates for the higher dose (24% and 40% respectively).

There was also a non-significant improvement in PFS favouring the lower dose (9 months v 7.2 months). The E2200 study (Giantonio, Levy et al. 2006) investigated 5mg/kg/week of bevacizumab combined with IFL chemotherapy as initial treatment for mCRC. This was a non-randomized single arm phase II study which reported impressive response rates and a median overall survival of 26.3 months. However two patients died and two developed bowel perforations amongst the 81 evaluable patients, suggesting increased toxicity. One randomized phase III trial (Giantonio, Catalano et al. 2007) has investigated the higher dose of bevacizumab (5mg/kg/week) in patients with previously treated colorectal cancer. The study investigators reported that there was no evidence of excess toxicity from the higher dose of bevacizumab compared with that reported for trials involving a lower dose of bevacizumab.

A dose response relationship was also observed in a trial of bevacizumab in metastatic renal cancer (Yang, Haworth et al. 2003). In this trial, placebo was compared with 3mg/kg or 10mg/kg of bevacizumab given every two weeks. Only the higher dose of bevacizumab showed a significant improvement in progression free survival compared to placebo. Studies in breast cancer (Miller, Wang et al. 2007) and non- small cell lung cancer (Sandler, Gray et al. 2006) which have used the higher dose of bevacizumab 10mg/kg every two weeks have also reported increased toxicity with no convincing benefit of increased efficacy.

A bevacizumab dose of 5mg/kg every two weeks, which has been widely used in clinical trials of colorectal cancer, represents a compromise between efficacy and toxicity and was the dose selected for our study.

1.3.3 End points for trials of anti-angiogenic drugs

According to conventional response assessments by radiological criteria (Therasse, Arbuck et al. 2000) the response rate for bevacizumab as a single agent is less than 5 percent.

However, unlike conventional cytotoxic chemotherapy, where response rate can be taken as a surrogate for decreased cell populations; the relationship between response rates and outcome is less clear-cut with regard to biological therapies, which have a cytostatic mode of action. Although the addition of bevacizumab to chemotherapy may improve response rates compared to combination chemotherapy alone by up to 10% (Hurwitz, Fehrenbacher et al. 2004), this does not necessarily result in increased overall survival (Grothey, Hedrick et al. 2008). Thus, response rate is not a valid primary end point in trials of biological agents such as bevacizumab.

Progression Free Survival (PFS) is defined as the time from first treatment, or enrolment in the case of clinical trials, until first evidence of disease progression, which is typically detected by routine radiological assessment and measured according to RECIST criteria (Therasse, Arbuck et al. 2000). Evidence from first line clinical trials in metastatic colorectal cancer (mCRC) of a correlation between PFS and overall survival (Tang, Bentzen et al. 2007) has led to the acceptance of PFS as an end point in clinical trials for patients with mCRC. Furthermore, with increasing lines of therapy there is an appreciation that any benefit from first line therapy may be obscured by subsequent lines of treatment. Based on these considerations progression free survival was chosen as the most appropriate primary end point for the biomarker trial described in this thesis.

1.4 Potential biomarkers of response to anti-angiogenic therapy in colorectal cancer

A biomarker can be 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 (Atkinson 2001). Biomarkers have many important applications in cancer, for example: screening, disease staging and prognosis. Increasingly, they have had an important role in the evaluation of compounds in early phase clinical trials where they can be used to guide drug dosing as well as acting as a surrogate for clinical outcome.

1.4.1 Predictive versus prognostic biomarkers

Prognostic biomarkers give an indication of the clinical outcome of a condition irrespective of treatment. In the curative setting, prognostic biomarkers can be used to estimate the likelihood of disease recurrence. In the metastatic setting, prognostic biomarkers correlate with progression free or overall survival. Although a prognostic marker can be used to select patients for a particular treatment they do not predict an individual's response to treatment. Before any biomarker can be accepted for clinical use it is important that technical analysis is reliable and difference in outcome between those who are positive or negative are statistically robust. In order to encourage full and transparent reporting of data, a guideline for reporting of tumour marker prognostic studies (REMARK) has been published by the National Cancer Institute- European Organisation for Research and Treatment of Cancer (NCI-EORTC) (McShane, Altman et al. 2005). This guideline recommends that information on: patient inclusion criteria, specimen characteristics and assay methods, study design, statistical methods, data and analysis methods are reported for each study in order to aid interpretation of results.

Predictive biomarkers can be used to stratify patients who are likely to respond to a particular therapy, as well as having a potential role in the early identification of therapeutic benefit. One of the earliest examples of a predictive biomarker was reported in patients with gastro-intestinal stromal tumours (GIST), when researchers discovered that a mutation in one of two receptor tyrosine kinases: (KIT or Platelet derived growth factor) indicated a subgroup of patients who responded to the tyrosine kinase inhibitor, imatinib (Heinrich, Corless et al. 2003). In breast cancer, it was recognised that amplification of the epithelial growth factor receptor-2 gene (HER-2) and concomitant over-expression of HER-2 surface expression, which occurs in approximately 20% of breast cancer patients, conferred an adverse prognosis. This poor prognosis was subsequently abrogated with the development of trastuzumab, an antibody targeting the extra-cellular domain of the Her-2 receptor (Vogel, Cobleigh et al. 2002). In contrast, the presence of K-ras or N-ras mutations predict a lack of response to anti-EGFR antibody therapy such as Cetuximab and are examples of negative predictive biomarkers (Heinemann, von Weikersthal et al. 2014).

Since their development ten years ago, the RECIST criteria for response assessment in clinical trials (Therasse, Arbuck et al. 2000) have been widely used to assess disease response in phase II and phase III trials. However, although these criteria are reliable and reproducible, they have a number of limitations and have been shown in several studies to be a poor indicator of response assessment to biological therapies such as imatinib (Benjamin, Choi et al. 2007) and bevacizumab (Grothey, Hedrick et al. 2008), which may have a more cytostatic mode of action.

Overall survival is the most reliable endpoint of any therapeutic intervention and has been the gold standard primary endpoint for randomised controlled trials evaluating new therapies. However such trials take many years to come to fruition and require large numbers of patients in order to show clinical significance. This has both financial and ethical implications.

For these reasons progression free survival is a more desirable end point and has been usually accepted as the primary endpoint in clinical trials in mCRC (Tang, Bentzen et al. 2007).

Potential biomarkers have been extensively investigated for their utility in the identification of early response to anti-angiogenic therapy. Putative biomarkers include: circulating markers, tissue markers, genetic markers and imaging markers of response. The purpose of this thesis was to investigate DCE- MRI biomarkers and circulating biomarkers prospectively in relation to progression free survival in patients with colorectal cancer. The evidence for circulating and tissue markers of angiogenesis to predict early benefit in colorectal cancer will shortly be reviewed. Imaging markers of angiogenesis will also be reviewed later in this thesis. Clinical measures of outcome such as hypertension and proteinuria have also been studied but evidence is inconclusive as there is a lack of prospective studies. Current evidence suggests that these toxicities are likely to reflect the pharmacodynamic properties of anti-angiogenic therapy rather than a biomarker of response. (Horsley, Marti et al. 2012). A recent analysis of 7 randomised controlled trials showed no relationship between the development of early hypertension and outcome (Hurwitz, Douglas et al. 2013). Results of the first prospective trial involving stratification of axitinib dosing based on blood pressure measurements have recently been reported (Rini, Melichar et al. 2015) and suggest that the relationship between drug exposure, hypertension and efficacy is more complex than anticipated. Although there was evidence of a longer PFS amongst patients who developed an increase in diastolic blood pressure; AUC (axitinib) and efficacy outcomes were not strongly correlated.

1.4.2 Tissue markers of angiogenesis

Microvessel Density (MVD), a measure of the number of vessels per high power microscopic field, has been widely used to quantify tumour angiogenesis in colorectal cancer. Immunohistochemical staining of pan-endothelial markers against Von Willebrand factor (factor VIII), CD31 and CD34 antigens have been used to identify tumour blood vessels. A meta-analysis of 22 studies investigating MVD in colorectal cancer (Des Guetz, Uzzan et al. 2006) demonstrated a significant association with relapse free survival (RR 2.32, 95%CI: 1.39-3.9) and overall survival (RR 1.44, 95% CI: 1.08-1.92). High levels of MVD in the primary tumour have been associated with an increased risk of haematogenous metastases (Tanigawa, Amaya et al. 1997). An elegant phase 1 study by Willet provided proof of concept by demonstrating reduced MVD in rectal cancer patients treated with bevacizumab (Willett, Boucher et al. 2004). Disappointingly, translational studies in colorectal cancer investigating MVD as a predictive marker of treatment response, following treatment with bevacizumab, have been negative (Jubb, Hurwitz et al. 2006).

Tissue VEGF expression in tumours was also been found to be prognostic for outcome in colorectal cancer, with respect to both relapse free survival and overall survival (Des Guetz, Uzzan et al. 2006). Other studies in colorectal cancer have shown reduced levels of tissue VEGF expression in the primary tumour compared with metastases (Lee, Chow et al. 2000). Furthermore levels appear to vary depending on metastatic site, with the liver showing significantly lower VEGF expression compared with other abdominal sites (Cascinu, Graziano et al. 2003).

Both MVD and VEGF measurements are limited by the fact that they are a static assessment of a dynamic process. Tissue measurements are further restricted by the need for biopsy samples and the possibility of sampling bias. In addition, it is possible that not all counted vessels have been functional.

To date, no studies have demonstrated that measurement of tissue VEGF is predictive of benefit to bevacizumab.

1.4.3 Circulating markers of angiogenesis to predict treatment response to bevacizumab

The analysis of circulating proteins which can be measured in peripheral blood has several advantages including low cost and the potential for repeated measurements over time. The most widely studied of these circulating proteins has been VEGF. Intuitively, VEGF levels might be expected to help stratify patients who would benefit from treatment with bevacizumab given its mechanism of action (MOA), as bevacizumab binds to and neutralises circulating levels of VEGF-A. However, results from randomised trials in advanced colorectal which have investigated baseline levels of VEGF as a predictive marker of response to treatment with bevacizumab (Goede, Coutelle et al. 2010) and cediranib (Jurgensmeier, Schmoll et al. 2013) have been disappointing. The largest study to investigate the relationship between baseline VEGF, bevacizumab and outcome was carried out by Hegde and colleagues who reviewed multiple phase III trials including patients with colorectal cancer (AVF2107), non-small cell lung cancer (AVAIL, E4599) and renal cell cancer (AVOREN, AVF2938) (Hegde, Jubb et al. 2012). Baseline circulating VEGF-A levels were measured by a multiplex enzyme-linked immunosorbent assay (ELISA) for 1816 patients and correlated with progression free survival (PFS) and overall survival (OS). The median levels of circulating VEGF-A were similar across different tumour types. Baseline levels of VEGF-A were defined as low or high according to median levels in each treatment arm of the different trials. In patients treated in the control arm of each trial, median VEGF-A levels were prognostic for OS but not PFS, with low levels of VEGF-A associated with improved survival.

However, patients who received treatment with bevacizumab showed no difference in median PFS or OS according to baseline VEGF-A levels. This study confirmed that baseline levels of circulating VEGF were prognostic for the cancers studied, although did not predict treatment benefit for bevacizumab.

The measurement of circulating VEGF in serum is complicated by the contribution from megakaryocytes and leucocytes both of which synthesize VEGF(Salven, Orpana et al. 1999); and platelet sequestration of VEGF(George, Eccles et al. 2000). For this reason, citrated plasma collection has been recommended as a more reliable method of measuring VEGF(Jelkmann 2001).

The most important receptor for VEGF-A is VEGFR-2, which is over expressed in both endothelial cells actively involved in angiogenesis and endothelial progenitor cells. A recent analysis of Horizon studies II and III found no prognostic association between sVEGFR-2 and either PFS or OS in patients with metastatic colorectal cancer treated with cediranib (Jurgensmeier, Schmoll et al. 2013). However, there are limited data regarding the prognostic or predictive value of soluble VEGFR-2 in colorectal cancer patients treated with chemotherapy and bevacizumab.

VEGF-D is a ligand for both the VEGFR-1 and VEGFR-2 receptor and is implicated in lymphangiogenesis and the development of lymphatic metastases (Karnezis, Shayan et al. 2012). A recent analysis of 207 serum factors in patients with metastatic colorectal cancer treated with cediranib identified that low levels of serum VEGF-D were associated with a better outcome (Jurgensmeier, Schmoll et al. 2013). However further studies are necessary to identify whether VEGF-D is a prognostic marker in colorectal cancer patients treated with bevacizumab.

Alternative signalling pathways such as the Angiopoietin (Ang)/Tie signalling pathway have also emerged as critical for angiogenesis, with the balance between Ang-1 and Ang-2 important in the determination of vascular phenotype (Cascone and Heymach 2012). In contrast to Ang-1 which has a stabilizing effect on tumour vasculature, Ang-2 functions as an Ang-1 antagonist by increasing vascular permeability and sprouting angiogenesis, but this is dependent on the presence of VEGF-A.

Low levels of serum Ang-2 have been proposed as a potential predictive marker of response to treatment with bevacizumab in patients with colorectal cancer (Goede, Coutelle et al. 2010). However, other groups have associated high levels of Ang-2 with reduced survival in metastatic colorectal cancer (Volkova, Willis et al. 2011) suggesting that Ang-2 may be a prognostic rather than a predictive biomarker. Elevated plasma levels of Tie-2 have been reported in patients with colorectal cancer, particularly in those patients with metastatic disease, compared with a control group (Chin, Greenman et al. 2004). The same study also observed that surgical resection of the primary tumour resulted in a reduction of serum Tie-2 receptor, in keeping with the tumour as the source of the receptor. There are limited data on the prognostic value of baseline levels of Tie-2 or Ang-1 in patients with metastatic colorectal cancer treated with bevacizumab and chemotherapy.

E-selectin is a cell adhesion molecule which is expressed on endothelial cells and contributes to the development of metastases by increasing the adhesion of cancer cells to endothelial cells. An important function of E-selectin appears to be in determining organ selectivity in cancer metastases (Gout, Tremblay et al. 2008). High levels of E-selectin have been reported in patients with metastatic colorectal cancer (Uner, Akcali et al. 2004), particularly in patients with liver metastases (Alexiou, Karayiannakis et al. 2001).

More recently, some researchers have proposed that low levels of E-selectin measured in patients with metastatic colorectal cancer prior to chemotherapy, were associated with an adverse prognosis (Inanc, Er et al. 2012).

Hepatocyte growth factor (HGF) is a potent mitogen for liver cells and the only known ligand for the c-MET receptor. The HGF/c-MET pathway promotes angiogenesis by promoting endothelial cell growth and also by induction of VEGF-A. Although this pathway is important in the malignant progression of a number of tumour types including colorectal cancer there are limited data on the prognostic impact of baseline levels of serum HGF in patients treated with bevacizumab and chemotherapy.

Dynamic changes in circulating markers of angiogenesis have also been proposed but not validated as potential indicators of response or resistance to anti-angiogenic therapy. Circulating angiocytokines which have been investigated in relation to colorectal cancer are shown in Table 6.

Circulating biomarker	Phase	N	Study description	End point	Results	Reference
sVEGFR-1	I/II	32	Locally advanced rectal cancer 5FU,Bevacizumab and radiotherapy	Radiological response (RR) and stage	Pre-treatment levels higher in non-responding patients Levels correlate with tumour stage	(Willett, Duda et al. 2009)
VEGF-A Ang-2	NA	34	Patients with mCRC received bevacizumab and combination chemotherapy	RR, overall survival (OS), progression free survival (PFS)	No correlation with VEGF-A and outcome Low pre-treatment Ang-2 levels correlated with improved RR, PFS and OS	(Goede, Coutelle et al. 2010)
VEGF-A	III	1816	4 RCTs of bevacizumab or placebo in colorectal, renal and NSCLC cancer	PFS or OS	Median VEGF-A levels prognostic for outcome in control group. No difference in outcome according to VEGF-A levels in bevacizumab treated group	(Hegde, Jubb et al. 2012)
IL-8	II	43	Patients with mCRC received FOLFIRI + bevacizumab 5mg/kg	PFS	Elevated IL-8 at baseline associated with shorter PFS	(Kopetz, Hoff et al. 2010)
Short VEGF-A isoform	III	398	IFL + bevacizumab or placebo	PFS or OS	No correlation between short VEGF-A isoform and outcome	(Jayson 2011)
VEGF sVEGFR-2 CEA	2 phase III studies	860 1422	FOLFOX/XELOX+cediranib or placebo mFOLFOX-6 + bevacizumab plus cediranib or placebo	PFS or OS	High baseline VEGF and CEA values associated with worse outcome in both studies. No relationship between baseline sVEGFR-2 and outcome CEA, VEGF and sVEGFR-2 not predictive	(Jurgensmeier, Schmoll et al. 2013)

Table 6: Circulating angiocytokines investigated in colorectal cancer studies.

1.4.4 Genetic markers of response to bevacizumab

There is no evidence that KRAS status is predictive of benefit in patients with metastatic colorectal receiving treatment with anti-angiogenic therapies. A sub-analysis of the AVF2107 study (Hurwitz, Yi et al. 2009) investigated treatment benefit from bevacizumab in patients according to KRAS status. However there was improved survival in both the control arm and treatment arm in the KRAS wild type subgroup of patients, suggesting KRAS status is a prognostic marker in colorectal cancer. This finding was not confirmed a recent phase III trial carried out by an Australian group (Price, Hardingham et al. 2011) who failed to identify a prognostic or predictive benefit for KRAS but found the presence of a BRAF mutation was associated with overall survival.

Polymorphisms in VEGF have also been proposed as candidate markers to predict response to bevacizumab treatment(Lambrechts, Lenz et al. 2013). However, although a variety of single nucleotide polymorphisms have been tested in different cancers including colorectal cancer, findings are inconsistent (Schneider, Wang et al. 2008; Gerger, El-Khoueiry et al. 2011; Koutras, Antonacopoulou et al. 2012). A recent pre-clinical study characterised altered transcription of VEGF dependant vascular (VDV) genes following ant-angiogenic therapy (Brauer, Zhuang et al. 2013). These researchers also investigated expression of VDV genes in pre-treatment biopsies of colorectal cancer patients who had participated in the NO16966 trial. They observed high levels of VDV gene expression associated with an improvement in PFS when patients were treated with bevacizumab. Although these data are promising they require confirmation in large prospective trials.

Researchers who carried out transcriptional analysis on samples from 265 patients with high grade serous ovarian cancer, identified 3 major subgroups of patients (Gourley, McCavigan et al. 2014).

A subgroup of patients characterised by repression of genes related to angiogenesis and up-regulation of immune genes had a longer progression free survival compared with 2 other subgroups where there was evidence of up-regulation of angiogenesis associated genes. The immune group was characterised using a 63-gene expression signature which was validated in an independent data set. This gene signature was tested on patients with high grade serous ovarian cancer who had received chemotherapy with or without bevacizumab within the ICON-7 trial. The patients in the immune group who received bevacizumab appeared to have shorter PFS and OS compared to those who received chemotherapy alone, whereas the addition of bevacizumab to chemotherapy in the other sub groups had no statistically significant effect on outcome. This gene expression signature requires validation in other data sets to confirm potential utility as a negative predictive biomarker in this patient population.

1.5 An introduction to imaging biomarkers

In contrast to circulating or tissue biomarkers, imaging is a non-invasive method for evaluating the tumour vasculature which facilitates both “in vivo” evaluation and longitudinal assessment. Functional imaging attempts to measure the pharmacodynamic effects of targeted therapies. Quantification of these effects could be a more sensitive indicator of response than standard RECIST assessments since targeted biological therapies such as bevacizumab have a predominantly cytostatic mode of action. Different functional imaging modalities have been used to investigate angiogenesis including: Computerised Tomography (CT), Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI), Ultra-sound (USS), Positron-emission Tomography (PET) and Diffusion Weighted Imaging (DWI). Each technique has strengths and weaknesses with regard to sensitivity, availability, reproducibility and areas of the body which can be measured.

CT imaging is widely available and has a high spatial resolution compared to other imaging modalities. Furthermore, in comparison to MRI, there is a direct relationship between contrast concentration and the change in tissue attenuation, measured in Hounsfield units (HU). Change in HU calculated between pre-contrast and post-contrast CT data can be used to assess tumour perfusion. Patients with colorectal cancer demonstrated a reduction in tumour perfusion measured by CT one week after treatment with bevacizumab (Koukourakis, Mavanis et al. 2007). Another simple measure which can be assessed using contrast CT is the proportion of enhancing voxels within a tumour or the enhancing fraction (EF). A disadvantage of CT imaging is the relatively high doses of radiation required which limits the number of repeat scans that can be safely acquired, particularly in the research setting.

Ultrasound imaging is cheap and widely available but the technique is highly operator dependent.

Dynamic contrast enhanced ultrasonography (DCE-US) involves doppler ultrasound with injection of low concentrations of micron-sized gas filled microbubble contrast agents to further enhance the vessel signal. Microbubbles are true intravascular contrast agents and can be used to measure tumour blood volume and flow, although capillary permeability cannot be assessed since microbubbles do not pass through endothelial cell membranes. Measurements such as “peak intensity”, “time to peak intensity”, area under the curve and slope of co-efficient wash out, have been assessed. A study involving thirty-eight patients with metastatic renal cancer treated with sunitinib (Lassau, Koscielny et al. 2010) measured parameters of perfusion and blood flow using DCE-US and identified that the parameter “time to peak intensity” was associated with both disease free and overall survival. The UK AXMUS-C trial (Axitinib Microbubble UltraSound in metastatic Colorectal cancer- Eudract 2011-002598-49) is a randomised phase II trial investigating contrast enhanced ultrasound in patients treated with axitinib or placebo following two previous lines of therapy for advanced colorectal cancer. This trial will test the potential for contrast enhanced ultrasound to predict response to axitinib by assessing early changes in tumour microvasculature in relation to RECIST criteria and overall survival.

A significant advantage of Positron Emission Tomography (PET) is that it allows whole body imaging. Although PET images have a lower spatial resolution than either MR or CT images they are highly quantitative due to the sensitivity to very small concentrations of tracer. However images are susceptible to motion, and reproducibility between scanners is limited due to the significant variation between individual scanners.

$H_2{}^{15}O$ is a freely diffusible positron-emitting tracer which specifically reflects tissue perfusion and blood flow in a given region of interest (de Langen, van den Boogaart et al. 2008). However, the very short half-life for this tracer restricts the technique to centres with appropriate cyclotron and chemical laboratory facilities.

The PET tracer 18-fluorothymidine ([^{18}F] FLT) is trapped in newly synthesized DNA and can be used to estimate cell proliferation. [^{18}F]FLT was used to investigate nineteen patients with recurrent malignant glioma who received treatment with bevacizumab and irinotecan chemotherapy (Chen, Delaloye et al. 2007). Those patients with evidence of a metabolic response, defined as a 25% reduction in the standard uptake value (SUV) of FLT following treatment, had a longer overall survival than non-responders: 10.8 v 3.4 months ($p = 0.003$). However, the utility of FLT as a tracer in metastatic colorectal cancer is limited by a low overall uptake and higher background activity in the liver due to hepatic glucuronidation.

Clinically, the most widely used PET tracer is ^{18}F-fluorodeoxyglucose (FDG) which is used routinely in the evaluation of a number of cancers, including colorectal cancer where it provides important information on staging and diagnosis of metastatic disease. A seminal study by Willet and colleagues (Willett, Boucher et al. 2005) investigated changes in FDG in a small series of patients with rectal cancer following treatment with bevacizumab and radiotherapy. The absence of any reduction in FDG uptake 12 days following treatment with bevacizumab at dosages of either 5mg/kg or 10mg/kg ($p=0.08$), suggested that FDG was unlikely to be of utility in early prediction of treatment response in colorectal cancer.

Since DCE-MRI does not require ionizing radiation, this lends the technique to be used for repeated assessments over time.

In colorectal cancer, correlation has been demonstrated between signal intensity measurements derived from DCE-MRI such as “time to peak enhancement”; “steepest slope of time intensity curve”(TIC) and micro-vessel density (Tuncbilek, Karakas et al. 2004). Specialist image analysis of varying complexity is required in order to extract biomarkers of tumour vascular function such as those representing plasma volume and vascular permeability.

However, although the assessment of vascular permeability (K^{trans}) using DCE-MRI has been widely accepted as a pharmacodynamic biomarker there are limited data relating this parameter to outcome (O'Connor and Jayson 2013). A detailed description of the technique of DCE-MRI and a review of trials relating DCE-MRI parameters to outcome are covered in a later section of this introduction.

Diffusion-weighted MRI (DWI-MRI) uses the diffusion properties of water within tissue to provide image contrast without the need for contrast injection. The technique can be used to give information regarding tissue cellularity and integrity of cellular membranes and water molecule displacement across distances of 1-20μm can be detected(Charles-Edwards and deSouza 2006). This displacement of water molecules can be quantified to provide a biomarker termed the apparent diffusion co-efficient (ADC). A limitation of the technique is the susceptibility to bulk motion artefact, although free breathing with multiple acquisitions is preferred over complex respiratory gating techniques(Padhani, Liu et al. 2009). An increase in the apparent diffusion co-efficient following chemotherapy treatment correlated with RECIST response in patients with colorectal cancer(Koh, Scurr et al. 2007). High pre-treatment ADC values have been shown to correlate with survival following treatment with bevacizumab in patients with recurrent glioma (Ellingson, Sahebjam et al. 2014). A description of the studies investigating DWI-MRI changes in relation to anti-angiogenic therapy follows in a subsequent section of this chapter.

1.5.1 Pros and cons of circulating versus imaging markers

The use of circulating biomarkers to evaluate response to therapy, have a number of potential advantages. They are relatively inexpensive when compared with imaging and far more simple to collect. This lends circulating markers to repeated assessments over a given period of time. In comparison, imaging requires complex equipment and often requires post-processing image analysis which is both time consuming and costly.

Certain imaging modalities such as CT also incur the additional risks of radiation exposure and intravenous contrast injection, making them less suitable for repeated assessments.

In order for a biomarker to be adopted into routine clinical practice it has to be validated as a surrogate end point for a clinically meaningful outcome. This can pose a challenge with circulating biomarkers due to the number of potentially confounding factors both known and unknown, due to the number of complex inter-related pathways at a cellular level. There also has to be a degree of confidence in the precision of the result. The potential for both inter and intra-observer variation with imaging techniques, as well as variation between individual scanners makes reproducibility between different centres challenging which has limited the implementation of multi-centre trials using imaging end-points. However, imaging permits “in-vivo” assessment and in contrast to circulating biomarkers, allows discrimination between a primary cancer and related metastases, as well as the study of tumour heterogeneity.

In reality, single biomarkers are unlikely to predict the complex phenotype of response and the combination of imaging and circulating biomarkers has the potential to provide complementary information regarding response which will be more sensitive to clinically validated outcome measurements such as progression free survival (PFS).

1.6 DCE MRI

Tumours blood vessels are highly disorganized, resulting in heterogeneity of blood flow and contributing to a hypoxic, acidotic tumour microenvironment. In addition to this functional abnormality, tumour vessels are also highly abnormal in structure. These abnormalities include defective or discontinuous basement membranes, abnormal endothelial cell growth and incomplete pericyte coverage. The resultant vessels are dilated and leaky with excessive branching. The ability to enhance microvascular permeability in tumours is largely due to the effect of tumour cell VEGF. The potency of VEGF A has been noted to be 50,000 times greater than that of histamine (Dvorak 2002)**.** This property is exploited in tracer kinetic modelling which allows calculation of a number of parameters that describe the structure and function of the tumour microvasculature. In this way, DCE-MRI has been widely used to investigate angiogenesis and response to anti-angiogenic treatment (O'Connor, Jackson et al. 2012).

The technique of DCE-MRI involves the injection of a low molecular weight, gadolinium chelate, contrast agent; followed by a rapid sequence of scans every few seconds. This allows contrast tracer to be tracked in the vasculature over a period of time. The contrast agent passes freely through permeable capillary endothelial membranes into the extra-vascular space, but does not enter the intracellular space. Gadolinium is a paramagnetic substance that greatly shortens T1 relaxation time of tissues producing an area of high-signal intensity on T1 weighted magnetic resonance images (Jackson, Parker et al. 2005). This enhancement can be used as a biomarker of change in the microvascular environment within the tumour. Where the baseline T1 values of the underlying tissue are known; the change in signal intensity can be used to calculate the concentration of gadolinium, allowing application of a range of pharmacokinetic analysis models. Table 7 details the standardised terms recommended for use in clinical trials (Tofts, Brix et al. 1999; Leach, Brindle et al. 2005).

Abbreviation	Definition	Units
IAUC	Initial area under the gadolinium contrast concentration curve	$mM.min^{-1}$
K^{trans}	Volume transfer constant plasma→EES	min^{-1}
k_{ep}	Volume transfer constant EES→plasma	min^{-1}
V_p	Tumour plasma volume/volume tissue	None
V_e	Volume of EES/volume of tissue	None
EF	Enhancing fraction	%
C_p	Tracer concentration in arterial blood plasma- input function	mM
C_t	Tracer concentration in tissue	mM

Table 7: Definition of clinical trial biomarkers used in DCE MRI

1.6.1 Semi-quantitative analysis

Semi-quantitative analyses of signal changes are simple to implement making them highly attractive in the clinical environment. However they have a number of limitations. One of the major problems in the application of semi-quantitative methodologies is the variation in signal intensity that can occur from day-to-day and from one scanning system to another. These variations are multi-factorial and include, for example, selection of scaling factors and amplifier gain. Due to the variation in absolute values of signal enhancement many semi-quantitative metrics are represented as ratios or rates of change against time, both of which will be independent of absolute measured signal intensity. Examples include *peak signal change*, *time to peak*, *the area under the enhancement curve* or, more commonly, *slope, or rate of enhancement and washout* (Jackson, Parker et al. 2005).

As a result of these variations, cross comparisons between patients, scanners or sites are difficult. Consequently, although some of these measurements have proven useful in the clinical diagnostic environment, they are not suitable for use in clinical trials evaluating anti-angiogenic or anti-vascular agents (Leach, Brindle et al. 2005). Nevertheless, a number of semi-quantitative approaches have found routine clinical application, particularly in the investigation of breast malignancy (Knopp, Weiss et al. 1999)

1.6.2 Calculation of (biomarker) parameters using pharmacokinetic modelling

Measurement of signal intensity allows the concentration of contrast media present in the tissue of interest to be calculated. Calculation of the contrast concentration requires knowledge of the underlying local T1 value in the tissue prior to enhancement. A variety of methods are used to map the baseline T1 values, the most common of these is the use of multiple flip angle gradient-Echo sequences (Jackson, Parker et al. 2005).

Flip angles are the name given to the Radiofrequency (RF) impulses used to change the orientation of the magnetic field around the tissue. The choice of flip angles is important in T1 imaging and gradient echo (GE) sequences generally use small flip angles. These are capable of providing sufficient accuracy in an acceptable imaging time. Once baseline T1 values have been estimated, these can be used to calculate the absolute concentration of gadolinium in each voxel within the image. This calculation can be performed for each point in a dynamic acquisition providing concentration/time course data for each voxel within the tumour over a period of minutes. Concentration-time curves are then mathematically fitted using an appropriate pharmacokinetic model and quantitative parameters are derived (see Table 7). This fitting process requires an estimate of the concentration changes of contrast over time occurring in arterial supply to the tumour, known as the arterial input function (AIF). In body imaging applications this is typically taken from the concentration of contrast in the aorta during and after contrast injection.

A range of pharmacokinetic models exists. These range from relatively simple two compartment models to models that include estimations of flow, blood volume, permeability surface area product of the capillary endothelium, the size of the extra vascular-extracellular space and regional blood flow. The choice of model is dependent on the microvascular parameters that are likely to be of interest given the specific mechanism of action of the therapeutic agent being studied. The terms and quantities derived by these models have been standardised by Tofts (Tofts, Brix et al. 1999) and these symbols are widely accepted as an international standard (Leach, Brindle et al. 2005).

More complex models are attractive in that they give greater physiological specificity but this must be balanced against higher susceptibility to signal-to-noise ratio variations, covariance fitting errors and other sources of potential error invoked by computationally complex models.

In addition to these limitations of pharmacokinetic analyses, most models are based on a series of assumptions about the behaviour of the contrast material and the underlying tissue. For example, there is an inherent assumption that leak of contrast into the intracellular space is negligible. However, there are animal studies (Noseworthy, Ackerley et al. 2002) that suggest this may be not be the case and that currently employed models may be an oversimplification.

1.6.3 Examples of pharmacokinetic models

The initial area under the curve (IAUC) (Evelhoch 1999) is simply the cumulative integral of the concentration of contrast within a region of interest over a given sampling period, typically 60 seconds (IAUC 60). The value obtained is dependent on the period over which the integration is performed. IAUC has been popular in drug development due to the fact that it can be calculated without curve fitting. However, there is evidence that this parameter is no more reproducible than pharmacokinetic parameters such as K^{trans} (Roberts, Issa et al. 2006) and also suffers from a lack of physiological specificity.

Tofts model (Tofts and Kermode 1991) is a simple 2 compartmental model developed for the investigation of Multiple Sclerosis (M.S.) plaques in the central nervous system and can be used to measures K^{trans} and $V_{e..}$ Although this model has been widely used in oncology, it is important to note that M.S. plaques are significantly less vascular than typical metastases. When this model is applied measured K^{trans} will be affected by changes in permeability and surface area product (PS), flow and V_p. This model assumes that K^{trans} is not flow limited and that none of the signal observed within the tumour is the result of intravascular contrast.

The extended Tofts model (Tofts 1997) (also known as the Kety model) allows calculation of K^{trans}, V_e and V_p.

This is the most widely used pharmacokinetic model for clinical trials of anti-angiogenic therapies (Morgan, Thomas et al. 2003; Miller, Trigo et al. 2005; O'Connor, Carano et al. 2009) since it represents an acceptable balance between physiological specificity; in addition to stability and accuracy of the estimated parameters. Where this model is employed, measured K^{trans} will be affected by changes in P.S. product and flow.

The St Lawrence and Lee model (St Lawrence and Lee 1998) allows calculation of flow in addition to the variables calculated in the extended Tofts model, and is the most physiologically specific of the models. However, the number of variables involved in the fitting process, make this model unstable and prone to error. This has restricted the use of this model in clinical practice. In this model K^{trans} represents a direct estimate of the P.S. product.

1.6.4 Choice of Arterial Input Function

The Arterial Input Function (AIF) is an estimate of the contrast concentration time course in the arterial supply to the tissue being studied. Measurement of the AIF is technically demanding for a number of reasons. The principal problem is that errors in the AIF will propagate significantly into the estimates of individual derived parameters during the pharmacokinetic analysis. Since the majority of DCE-MRI studies now use a bolus injection of contrast, the rate of change of contrast concentration in the feeding vessels is extremely rapid, particularly during the first minute after injection.

Accurate estimation of the AIF demands high sampling rates (typically 2-4 seconds), which are difficult to achieve in most imaging protocols. In abdominal studies the AIF is typically taken from the aorta but great care must be taken to avoid inflow artefacts within the imaging volume, which can also give rise to significant errors. Although most investigators favour direct measurement of the arterial input function, in practice this is not always achievable.

An alternative standardised “population AIF” derived from measurements in a large number of individuals can be used for all cases. These population AIFs are widely used and allow a lower temporal resolution sequence to be designed to study the changes of concentration within the tumour tissue. A recent study of 25 patients with lung and liver metastases showed a substantial improvement in the reproducibility of liver lesions when a patient-specific arterial input was derived compared with that obtain from a normal population data set (Ashton, Raunig et al. 2008). However, the use of population AIF values is based on a number of assumptions about the biological behaviour of the contrast bolus. Most important of these is the assumption that the shape, concentration and duration of the AIF will not be affected by the treatment under investigation. It is also assumed that the rate of injection and the passage of contrast into the systemic circulation will be constant from study to study. Although a power injector helps to reduce differences in injection time there may still be differences related to physiological parameters such as blood pressure, renal function and cardiac output between scans and patients which are more challenging to correct.

1.6.5 DCE- MRI biomarkers in clinical trials

Several phase I studies have demonstrated a dose-response relationship between escalating doses of anti-angiogenic agent and calculated DCE-MRI parameters such as K^{trans} and IAUC (Morgan, Thomas et al. 2003; Liu, Rugo et al. 2005).

As a consequence, DCE-MRI parameters such as K^{trans} and IAUC are accepted pharmacodynamic biomarkers which are widely used in phase 1 trials of anti-angiogenic compounds. In this way, they have been used to support the proposed mechanism of action of the drug, as well as assisting in optimum dose selection and scheduling. However, no phase 1 trials incorporating DCE-MRI parameters have demonstrated any evidence of correlation with outcome (O'Connor and Jayson 2013).

Reasons for this include the heterogeneity of disease type studied in early phase trials, extensive use of prior therapies in this population, small number of patients typically recruited to such studies and lack of standardisation between DCE-MRI protocols in different centres.

Larger phase II trials in a single disease group have also investigated DCE-MRI biomarkers in patients treated with anti-angiogenic drugs and these are summarised in Table 8. As can be seen from the table, there are a large variety of tumour types which have been studied and patient numbers are small. These studies have investigated the relationship between variety of MRI parameters and validated trials end points such as response rate and survival.

Phase II	Drug (Reference)	Tumour	No. Pts	DCE MRI Parameters studied	Result
Non randomized	bevacizumab and erlotinib (de Langen, van den Boogaart et al. 2011)	Non small cell lung cancer	28	K^{trans} K_{trans} SD	No association between K^{trans} and PFS. K^{trans} SD > 15% predictive for disease progression.
Non randomized	sorafenib (Kelly et al, 2011)	Non small cell lung cancer	26	K^{trans} K_{ep} V_e	No correlation of MR parameters with response. K_{ep} change from baseline correlated with PFS and OS.
Non randomized	sunitinib (Machiels et al, 2010)	Squamous head & neck	4	K_{trans} V_p	K^{trans} elevated at 6-8 weeks in non responding patient
Non randomized	sunitinib (Zhu et al 2009)	Hepatocellular carcinoma	34	K^{trans} K_{ep}	Correlation between extent of decrease in K^{trans} and radiological response, with larger reduction in responding patients.
Randomized	sorafenib (Hahn, Yang et al. 2008)	Renal cell cancer	44	IAUC 90 K^{trans} V_p	IAUC90 and K^{trans} showed dose related changes. Baseline K^{trans} split according to median correlated with PFS
Non randomized	sorafenib (Flaherty, Rosen et al. 2008)	Renal cell cancer	17	K^{trans}	Baseline K^{trans} and % reduction K^{trans} correlated with TTP
Non randomized	AZD2171(cediranib) (Batchelor, Sorensen et al. 2007)	Glioblastoma	16	T1 post contrast ADC K^{trans} V_e	Demonstrated a vessel normalization window and that this was reversible if drug interrupted. No correlation between radiological response and outcome. The sustained reduction in V_e felt to represent decreased oedema*.
Non randomized	bevacizumab (Wedam, Low et al. 2006)	Inflammatory Breast Cancer	21	K^{trans} K_{ep} V_e	No differences in MR parameters for responders versus non responders.
Non randomized	bevacizumab (Kreisl, Zhang et al. 2011)	Recurrent anaplastic glioma	31	ETV K^{trans}	Reduced ETV at 4D correlated with reduction in PFS
Non randomized	sorafenib (Hsu, Shen et al. 2011)	Hepatocellular cancer	31	K^{trans}	High baseline K^{trans} correlated with tumour response Reduction in percentage change K^{trans} D14 correlated with PFS and OS
Non randomized	bevacizumab (De Bruyne, Van Damme et al. 2012)	Colorectal cancer	19	K^{trans}	>40% reduction in K^{trans} associated with improvement in PFS

Table 8: Phase II trials of anti-angiogenic drugs incorporating DCE MRI.

*Further publication (Sorensen, Batchelor et al. 2009) reported that a reduction in K^{trans} at 24 hours correlated with PFS and OS

1.6.5.1 DCE MRI parameters and relationship to radiological response

Trials that have attempted to correlate MR parameters with radiological response according to RECIST have shown inconsistent results. A study of patients with hepatocellular cancer treated with sunitinib showed that a greater reduction in K^{trans} from baseline correlated with response. However, other studies (Wedam, Low et al. 2006; Batchelor, Sorensen et al. 2007), have shown no relationship between response rates and MR parameters.

1.6.5.2 DCE MRI parameters and correlation with survival

Baseline K^{trans} has shown promising results as a predictor of PFS. This was initially described in a pilot study of patients treated with sorafenib renal cell cancer (Flaherty, Rosen et al. 2008) and confirmed in a later randomised trial (Hahn, Yang et al. 2008). Follow up from a small study in glioblastoma (Sorensen, Batchelor et al. 2009) has shown K^{trans} at 24 hours related to both PFS and OS. Change in K_{ep}, from baseline to day 14, has been shown to correlate with both PFS and OS in NSCLC patients treated with sorafenib (Kelly, Rajan et al. 2011). These results are encouraging, particularly for kinase inhibitors, and larger confirmatory studies are needed.

1.6.5.3 DCE-MRI parameters selected for the Travastin-1 study

Whole tumour volume (WTV) as measured by DCE-MRI provides a 3-dimensional estimate of disease volume compared to traditional RECIST criteria, which are based on measurement in a single dimension (Therasse, Arbuck et al. 2000). It was therefore hypothesized that this simple metric may provide useful prognostic information that would correlate with other measures of disease burden in our patient cohort.

Another parameter which can easily be extracted from DCE-MRI data is the enhancing fraction (EF); the proportion of tissue within a tumour that shows any evidence of contrast enhancement.

Studies in patients with ovarian cancer (O'Connor, Jayson et al. 2007) and carcinoma of the cervix (Donaldson, Buckley et al. 2009) demonstrated that a high enhancing fraction as measured using Computerised Tomography prior to therapy, was associated with a poorer outcome. In glioma, a highly angiogenic tumour, EF assessed by DCE-MRI has been shown to correlate with tumour grade, with increased contrast enhancement at higher tumour grades (Mills, Soh et al. 2009). Consequently, there has been interest in establishing whether EF measured by DCE-MRI at baseline provides prognostic information regarding response to anti-angiogenic therapy in patients with metastatic colorectal cancer.

The Initial area under gadolinium contrast agent concentration-time curve (IAUC) is a semi-quantitative parameter which reflects contrast uptake in the tissue of interest over a given time; typically 60 seconds. The parameter has been widely used in early phase drug trials since it is relatively easy to implement and reproducible. Although there is evidence from early phase trials that reductions in IAUC reflect the pharmacodynamic activity of anti-angiogenic compounds (Hahn, Yang et al. 2008), data are limited on whether baseline measures have any prognostic relevance.

The parameter K^{trans}, estimated by fitting a pharmacokinetic model to the gadolinium time course curve, is thought to be more physiologically specific than IAUC, since it can reflect ,capillary surface area and flow (depending on the model used). K^{trans} has been widely studied in early phase clinical trials of anti-angiogenic therapies, where a reduction has been proposed to reflect the process of vessel normalization (Willett, Boucher et al. 2004; Batchelor, Sorensen et al. 2007). However, whether K^{trans} is purely a marker of pharmacodynamic response (Liu, Rugo et al. 2005), or holds potential to aid selection of patients more likely to benefit from anti-angiogenic therapy, remains undetermined. An improvement in progression free survival in patients with high baseline K^{trans}, dichotomised according to median values, was reported in patients with renal cancer treated with sorafenib (Hahn, Yang et al. 2008).

Similarly, a research group investigating sorafenib in patients with hepatocellular cancer identified that higher baseline K^{trans} values were associated with an improved radiological response (Hsu, Shen et al. 2011). However to date, no large imaging studies have investigated the significance of baseline K^{trans} in patients with metastatic colorectal cancer treated with bevacizumab and chemotherapy.

The volume of extracellular extravascular space per unit volume of tissue (V_e) is a parameter derived from most pharmacokinetic models which is thought to represent the interstitial space. However, the relevance of this parameter in relation to treatment with anti-angiogenic drugs is unknown.

The blood-plasma volume (V_p) is typically the least reproducible of the DCE-MRI parameters. This is partly due to technical demands in the DCE-MRI acquisition (O'Connor, Jackson et al. 2012) but also partly reflects the influence of patient-related variables such as hydration status, blood pressure and haematocrit, for example, which are not accounted for in the parameter estimation. This withstanding, researchers investigating patients with renal cancer treated with sorafenib identified that a high baseline V_p was associated with a longer progression free survival (Hahn, Yang et al. 2008). We hypothesize that baseline V_p could provide prognostic information regarding tumour blood volume which may relate to treatment benefit with chemotherapy and bevacizumab.

Calculation of T1, the pre-contrast longitudinal relaxation time, is a routine part of DCE-MRI data acquisition; although few studies have reported on the significance of this parameter in relation to treatment. Previous work from our group (O'Connor, Carano et al. 2009) suggested that this parameter may reflect tumour oedema and therefore could provide additional information regarding the tumour microvascular environment which may be of relevance to treatment response.

1.7 Diffusion-weighted MRI (DWI-MRI)

Diffusion-weighted MRI (DWI-MRI) uses the diffusion properties of water within tissue to provide image contrast. This movement of water molecules in tissues occurs by Brownian motion and is sensitive to the surrounding cellular environment. The diffusion characteristics of a tissue are influenced by both the vascular compartments and the extravascular compartments. However it is the signal from the extracellular compartment which is related to both tissue cellularity and the tortuosity of the extracellular space. Therefore although the displacement of pure water molecules at 37°C over a period of 50-milliseconds is approximately 30μm (Norris 2001), the observed or "apparent" diffusion of water in vivo is significantly less than this.

The sensitivity of a DWI-MRI sequence is characterised by the b-value which determines both the strength and duration of the diffusion gradients: with clinically relevant b-values ranging from 0-1000. The apparent diffusion coefficient (ADC) is calculated using linear regression on a pixel by pixel basis to form an ADC map. It is the slope of the signal decay which provides the ADC value, thus rapid signal decay, will produce a steeper slope and a corresponding high ADC value. In general, increased cellularity in malignant lesions restricts water motion due to a reduced extracellular space, resulting in lower ADC values. Following successful treatment, an increase in the ADC value has been observed in several malignancies including brain tumours (Moffat, Chenevert et al. 2005), breast cancer(Jensen, Garzon et al. 2011) and liver metastases secondary to colorectal cancer (Koh, Scurr et al. 2007).

In some tissues such as the brain and muscle, diffusion is directionally dependent or anisotropic. Although most other tissues are assumed to be isotropic (independent of direction), it is safer to assume there may be some directional dependency. For this reason it is recommended that at least three gradient directions are used and then averaged to give a mean value(Padhani, Liu et al. 2009).

Since tumour cell death and changes in tumour vasculature typically precede a measurable reduction in tumour size, DWI-MRI could be used as an early biomarker to predict treatment outcome following treatment with anti-vascular therapies such as bevacizumab.

1.7.1 Studies of DWI-MRI and anti-angiogenic therapies

A preclinical evaluation of DWI-MRI was carried out in rats implanted with rhabdomyosarcoma tumours, following treatment with the vascular disrupting agent combretastatin A4 phosphate (Thoeny, De Keyzer et al. 2005). A significant reduction in the perfusion-associated values of ADC, calculated by subtraction of high ADC values from low ADC values, was noted at one hour and six hours after treatment. These ADC values had risen two days following treatment and had increased beyond baseline values at nine days following treatment. Corresponding histopathology of tumour specimens showed that there was evidence of vessel constriction in the tumour periphery at one hour and that there was evidence of tumour oedema at the 6 hour time point. At two days, only a small rim of viable tumour tissue remained and only normal vessels were present at the tumour periphery. However by nine days, there was evidence of increased blood vessel and tumour growth. This important study provided a time course for the activity of vascular disruptive therapies and validation of DWI-MRI measurements as a potential early pharmacodynamic biomarker.

A study including twelve patients with hepatocellular cancer treated with the tyrosine kinase inhibitor sorafenib evaluated the time course of DWI-MRI changes in lesions during treatment. There was evidence of an initial decrease in the ADC at 2-4 weeks after treatment which subsequently increased following an average of 9.9 weeks of treatment (Schraml, Schwenzer et al. 2009). After 3 months there was evidence of reduction in ADC, consistent with increased diffusion restriction and disease progression.

The time course of DWI-MRI changes, DCE-MRI and plasma VEGF-A levels was assessed in ten patients with intra-abdominal metastases due to metastatic renal cancer (Desar, ter Voert et al. 2011).

Patients received treatment with sunitinib, a tyrosine kinase inhibitor, and images were acquired at baseline, three days and nine days following treatment. There was evidence of a significant increase in ADC values from baseline at day 3 (p=0.015) and a reduction in ADC values back to baseline values by day 10 (p=0.001).There were no significant changes in DCE-MRI parameters. Although an increase in plasma in VEGF-A was observed at day 10 this did not correlate with DWI-MRI measurements.

Jain's group investigated DWI-MRI in patients with recurrent or progressive malignant brain tumours treated with bevacizumab alone, or in combination with chemotherapy (Jain, Scarpace et al. 2010). Those patients whose tumours had progressed showed a reduction in the ADC value which was significant at 3 months (p=0.023), whereas those patients with tumours which had not progressed, showed stable or increased ADC values at 3 months (p=0.025).

Histogram analysis of pre-treatment ADC values prior to bevacizumab treatment has been correlated with survival in patients with brain tumours by Pope and colleagues (Pope, Kim et al. 2009; Pope, Lai et al. 2011). This group used a two- normal distribution mixture to fit histograms of ADC values. Values from the lower of the two distributions (ADC_L) dichotomised about the mean, were predictive of a longer median progression free survival (PFS) in patients who had received treatment with bevacizumab. Although a high mean ADC_L value pre-treatment was associated with a 6.6 fold increase in PFS, compared with a 2.4 fold increase in PFS for patients who had a low mean ADC_L value (Pope, Kim et al. 2009), this difference in survival according to dichotomised ADC_L values was not statistically significant.

A further retrospective study was conducted by the same group in patients with newly diagnosed glioblastoma who had received concurrent chemo-radiotherapy treatment with bevacizumab as initial treatment or concurrent chemo-radiotherapy followed by treatment with bevacizumab when the disease had progressed (Pope, Lai et al. 2011).

A high pre-treatment tumour ADC_L value was associated with a longer PFS in patients who received bevacizumab at disease progression (delayed) compared with the group who received initial treatment with bevacizumab (p=0.0046, log rank test). Patients with a low tumour ADC value pre-treatment showed no difference in survival according to treatment with initial versus delayed bevacizumab. The association between high pre-treatment ADC_L tumour values and longer survival was observed in a more recent retrospective study from the same group involving 130 subjects(Ellingson, Sahebjam et al. 2014). Patients who had received chemotherapy alone in their treatment were compared against those who had received treatment with both bevacizumab and chemotherapy, according to pre-treatment ADC_L values. There was a significant difference in overall survival in favour of the cohort of patients with a high pre-treatment ADC_L value who received bevacizumab compared with chemotherapy alone (376 days versus 255 days, HR 0.48, p=0.0016). In contrast, there was no difference in survival between the different treatments for patients with a low pre-treatment ADC_L value. Furthermore, within the group of patients who received bevacizumab, overall survival was significantly improved for those patients with a high versus low pre-treatment ADC_L value (376 days versus 255 days, HR 0.49, p=0.0016). These data would indicate that pre-treatment ADC_L holds promise as a predictive biomarker for response to bevacizumab in patients with recurrent glioma. However, larger prospective studies are required before this biomarker could be validated for widespread use.

1.7.2 Studies investigating DWI-MRI in colorectal cancer

Several groups have investigated whether DWI can predict early response to therapy in patients with liver metastases secondary to colorectal cancer, these studies are summarised in Table 9. A consistent finding is that high mean ADC measured prior to treatment with chemotherapy predicted a poor treatment response (Koh, Scurr et al. 2007; Cui, Zhang et al. 2008; Tam, Collins et al. 2013). A high pre-treatment ADC would seem to be associated with a more aggressive tumour phenotype.

This could be due to the presence of tumour necrosis which would result in poor tumour perfusion and a hypoxic environment which would be more resistant to the effects of chemotherapy. Serum VEGF-A levels have been found to correlate with mean ADC values in patients with liver metastases secondary to colorectal cancer (Heijmen, Ter Voert et al. 2013). However, an increase in ADC values was observed in tumours responding to chemotherapy following assessments at 12 weeks (Koh, Scurr et al. 2007) and 6 months (Anzidei, Napoli et al. 2011). This would be consistent with the significant inverse relationship between cellularity and ADC (Chen, Liu et al. 2013; Chen, Zhang et al. 2014) documented in lung cancer and primary brain tumours. Therefore, lesions responding to chemotherapy would be less cellular following chemotherapy, leading to higher ADC values in responding lesions. Currently there no accepted standards in measurement or analysis methods for the use of DWI-MRI which has constrained the use of the technique in large multi-centre trials, although recommendations for standardisation of the technique in trials have been proposed (Padhani, Liu et al. 2009). At the time of writing, there are no large published series correlating DWI-MRI with clinical outcome in patients with metastatic colorectal cancer.

Phase	Drug (Reference)	Tumour	No. Pts	DWI parameter studied	Result
Non randomized	Bevacizumab Capecitabine Oxaliplatin (Anzidei, Napoli et al. 2011)	Colorectal cancer liver metastases	18	ADC	No correlation between baseline ADC and RECIST response.
Non randomized	Chemotherapy (Mungai, Pasquinelli et al. 2013)	mixed tumours	33	ADC	No correlation between baseline ADC and RECIST response.
Non randomized	Oxaliplatin Capecitabine (Koh, Scurr et al. 2007)	Colorectal cancer liver metastases	20	ADC	High pre-treatment ADC predicted a poorer response to chemotherapy. Responding lesions had a significant increase in ADC at the end of 12 weeks of chemotherapy.
Non randomized	Unspecified chemotherapy (Cui, Zhang et al. 2008)	Colorectal and gastric cancer liver metastases	23	ADC	High mean pre-treatment ADC predicted a poorer response to chemotherapy. Responding lesions showed an early increase in ADC following treatment.
Retrospective	Chemotherapy, surgery and radio-frequency ablation (RFA) (Tam, Collins et al. 2013)	Colorectal cancer liver metastases	102	ADC	Higher mean pre-treatment ADC in non-responding patients. No relationship between pre-treatment ADC and PFS or OS.

Table 9: Trials incorporating DWI and colorectal cancer liver metastases

1.8 Assessment of heterogeneity using imaging biomarkers in patients with multiple colorectal cancer liver metastases

Knowledge of the gene mutations that drive cancer has led to the development of a large number of mechanism-based therapeutics (MBT) which to date, have been evaluated in traditionally designed phase I-III clinical trials. The cost of taking a drug to market has increased significantly and there is a clear need to improve trial design so that fewer patients are exposed to ineffective drugs and decision making for new agents is accelerated.

The development of MBT has encouraged the parallel evolution of biomarkers that, in early clinical trials, provide proof of mechanism and principle. Imaging biomarkers are particularly attractive as they allow interrogation of the whole tumour, repeated measurements over time and support studies of inter- and intra-tumoural heterogeneity (Li, Wilmes et al. 2005; Asselin, O'Connor et al. 2012; Fisher, Pusztai et al. 2013). Genetic heterogeneity between primary tumours and related metastases has been described in renal (Gerlinger, Rowan et al. 2012) and colorectal cancers (Jones, Chen et al. 2008), yet the potential for this heterogeneity to be used to accelerate early phase clinical trials has largely been ignored. Focusing on measures of drug activity i.e. pharmacodynamic and proof of principle biomarkers, the question is whether imaging data derived, for example, from forty patients who each have a single liver metastasis provide the same amount of data as twenty patients who have two or more liver metastases. To date, this question has not been addressed.

Although heterogeneity between metastases in individual subjects has been less extensively studied, there is evidence from colorectal cancer liver metastases to suggest that phenotypic and genetic heterogeneity exist between metastatic sites within the same patient (Jones, Chen et al. 2008). This is supported by a recent retrospective analysis of the CAIRO and CAIRO II trials which demonstrated mixed response to therapy in 36% of patients with multiple metastases, associated with a decreased median survival of 23.7 months compared to 36 months in patients with homogeneous response.

Goasguen and colleagues (Goasguen, de Chaisemartin et al. 2009) reported significant variation in treatment response in 64% of primary tissue cultures derived from different metastases within the same patient. This was also associated with considerable inter-metastatic heterogeneity in levels of gene expression. The authors concluded: “Our results demonstrate inter-metastatic and intra-metastatic heterogeneity suggesting that pre-therapeutic analysis of a single tumour biopsy is likely to lead to a misinterpretation of sensitivity to anticancer treatment”. This conclusion poses significant potential problems for the development of targeted molecular therapies and for the development of personalised treatment strategies since multiple tissue biopsies are clearly unacceptable and impractical.

As part of this thesis, and using the data collected from the Travastin-1 trial, the hypothesis was tested that standard DCE-MRI and DWI-MRI techniques are capable of detecting differences between metastatic deposits in different patients and between individual metastatic deposits in individuals. The primary objective was to determine whether current imaging biomarkers contain sufficient information to detect these differences; the aim being to support development of imaging biomarkers that could underpin personalised treatment strategies.

1.9 Aims and hypotheses

The aim of the Travastin-1 trial is to identify a biomarker signature which will help to identify patients with metastatic colorectal cancer who may benefit from bevacizumab in addition to chemotherapy. The purpose of this thesis was to evaluate DCE- MRI biomarkers and circulating biomarkers prospectively in patients with metastatic colorectal cancer receiving treatment with chemotherapy and bevacizumab, with the specific aims:

1. To assess the clinical outcome in the Travastin-1 population in relation to known prognostic factors in metastatic colorectal cancer.
2. To establish the reproducibility of pre- treatment DCE-MRI and DWI parameters in a population of patients with metastatic colorectal cancer.
3. To test the hypothesis that pre-treatment K^{trans} and EF are prognostic biomarkers for progression free survival in patients with metastatic colorectal cancer treated with bevacizumab and mFOLFOX-6 chemotherapy.
4. To test the prognostic potential of additional DCE-MRI and DWI parameters in relation to clinical outcome.
5. The evaluation of selected angiocytokines obtained pre-treatment as biomarkers for clinical outcome in patients with metastatic colorectal cancer treated with bevacizumab and mFOLFOX-6 chemotherapy.
6. To evaluate dynamic changes in DCE-MRI and DWI parameters following treatment with bevacizumab in relation to clinical outcome.
7. To evaluate dynamic changes in angiocytokine levels following treatment with bevacizumab in relation to clinical outcome.
8. To investigate any correlations between angiocytokines and imaging parameters before and after treatment with bevacizumab.
9. To investigate heterogeneity within the subpopulation of patients in the Travastin-1 trial with multiple liver metastases.

Chapter 2: Methodology

2.1 Study design

The Travastin-1 study, scientific title “An assessment of imaging and circulating biomarkers in patients with metastatic colorectal carcinoma treated with the anti-vascular endothelial growth factor (anti-VEGF) antibody, bevacizumab”, was a phase IV biomarker study, (EudraCT number 2009-011377-33) sponsored by The Christie NHS Foundation Trust. This trial was open to recruitment from November 2009 until October 2012. The aim of the trial was to investigate baseline and longitudinal treatment effects of standard of care chemotherapy (oxaliplatin and fluoropyrimidine), in combination with bevacizumab, on a panel of tissue, circulating and imaging biomarkers; in patients with untreated metastatic colorectal cancer. Ethical approval was given by the NHS Health Research Authority National Research Ethics Service, North West Greater Manchester Central ethics committee (reference 09/H1008/99). All patients had given written informed consent to participate in the study, which was carried out in accordance with standards of Good Clinical practice (GCP).

In my capacity as clinical research fellow for the trial, I was responsible for patient recruitment, screening patients, overseeing collection of biomarker samples and supervision of DCE-MRI scans. An overview of the study design and biomarker collection is shown in Table 10.

Time point	Assessment	Treatment
Baseline 1	Circulating DNA DCE-MRI & DWI Circulating biomarkers	
Baseline 2	DCE-MRI & DWI Circulating biomarkers CECs	
Day 0		bevacizumab 10mg/kg
Day 2	DCE-MRI & DWI	
Day 7	Circulating biomarkers CECs	
Day 14	DCE-MRI & DWI Circulating biomarkers	bevacizumab 2.5mg/kg/week oxaliplatin and fluoropyrimidine*
Day 21	DCE-MRI & DWI Circulating biomarkers CECs	
Week 6	Circulating biomarkers[#]	
6 months	DCE-MRI & DWI	
Disease progression	DCE-MRI & DWI Circulating biomarkers CECs	

Table 10: Outline of Travastin-1 study design and biomarker collection

*Treatment was continued every 2 weeks until disease progression

[#]Circulating biomarkers were collected every 6 weeks until disease progression

2.1.1 Baseline assessments

Patients underwent DCE MRI and DWI scans and blood tests to allow assessment of circulating biomarkers prior to and following 10mg/kg of bevacizumab. This was to evaluate independently the effect of single agent bevacizumab on circulating and imaging biomarkers prior to the addition of cytotoxic chemotherapy. Two baseline measurements for each patient of DCE MRI, DWI MRI and circulating biomarkers were acquired at least 48 hours apart in the 2 weeks prior to treatment. This was done so that the baseline measurement variability in tumour vascular characteristics that occur within a lesion at two given time points could be estimated and thereby, the significance of any drug-induced effects determined.

Two 6ml blood samples in EDTA were collected from patients on the Travastin-1 trial in the 14 days prior to starting treatment with bevacizumab and at least forty-eight hours apart. Samples were taken on the same day patients attended for MRI scans or other trial-related procedures. This was to quantify the variability in levels of each biomarker between baseline visits within a given patient. Plasma analysis was carried out using a multiplex ELISA method described in this section. Each sample was analysed in duplicate and pre-treatment values for the current analysis were calculated by obtaining an average measurement from the two pre-treatment visits. Average values which were above or below the limit of quantitation were excluded from the analysis.

The imaging parameters included in the analysis included:

- Whole tumour volume (WTV) - a measure of the individual lesion size
- T1- measured prior to contrast

- Enhancing fraction (EF) - the proportion of the tumour with evidence of contrast uptake
- IAUC-60- the initial area under the gadolinium time course concentration curve at 60 seconds.
- K^{trans} – volume contrast transfer co-efficient
- V_e- the proportion of contrast in the Extravascular Extracellular Space
- V_p- tumour plasma volume
- ADC- the apparent diffusion co-efficient

2.1.2 Dynamic assessments

2.1.2.1 DCE-MRI and DWI-MRI

Further imaging was carried out 48 hours after bevacizumab treatment to investigate early drug-induced changes in tumour microvasculature. At fourteen days, and prior to receiving bevacizumab 2.5 mg/kg weekly, together with oxaliplatin and fluoropyrimidine chemotherapy, patients again underwent imaging which served as a baseline for treatment combination effects which were again assessed 7 days after the combination of chemotherapy and bevacizumab, at day 21.

The timing of DCE-MRI assessments was informed by an earlier pilot study carried out at our institution (O'Connor, Carano et al. 2009). Additional DCE-MRI and DWI assessments were carried out at 6 months, for patients who remained on study and at the time of progression of the patient's disease.

2.1.2.2 Circulating angiocytokines

Additional blood samples were collected at:

- Day 7: 7 days after bevacizumab dosing
- Day 14: prior to chemotherapy and bevacizumab dosing
- Day 21: 7 days after chemotherapy and bevacizumab dosing

When possible, samples were taken on the same day that patients attended for DCE-MRI scans. Sample collection continued every 6 weeks until the point where the patient's disease progressed, to investigate biomarkers of resistance to angiogenesis, and to determine the extent that changes in such biomarkers might precede radiological progression of disease. A final sample was also collected after documented progression of disease according to RECIST criteria.

Blood samples to measure platelet count, serum LDH and CEA were collected within two weeks prior to any cancer therapy as part of the Travastin-1 trial.

2.2 Patient Eligibility

This study was open to patients with histologically confirmed colorectal cancer and at least one lesion ≥ 30mm suitable for DCE-MRI assessment, with no contra-indications to MRI scanning. Prior therapy for advanced disease was not permitted but previous adjuvant therapy was allowed providing the last dose was administered more than 12 months before entry into Travastin-1. Patients also had to have adequate Eastern Co-Operative Group (ECOG) performance status (0 to 2), a minimum of 18 years of age and a life expectancy of more than 12 weeks. Signed informed consent was required as well as adequate bone marrow, hepatic and renal function (with < 2+ protein on urine dipstick). An INR ≤1.5 and aPTT≤1.5 x ULN within 7 days of study treatment was also stipulated.

Criteria that excluded patients were the presence of brain metastases or spinal cord compression, uncontrolled inter-current illness, pregnant or breast-feeding women and surgery, significant traumatic injury or radiotherapy within 4 weeks prior to first treatment. No previous VEGF inhibitor treatment was permitted nor was evidence of poorly controlled hypertension (defined as sustained blood pressure >150/100mmHg despite anti-hypertensive therapy), clinically significant cardiovascular disease, previous CVA or TIA within the last 6 months prior to trial entry, haemorrhagic disorders, regular NSAID use (including use of more than 325mg/day of Aspirin), use of warfarin at any dose or therapeutic anticoagulation commenced within 4 weeks of trial entry.

Other exclusions included previous malignancies within the past 5 years, participation in a clinical trial within the previous 30 days prior to study entry, known dihydro-pyrimidine dehydrogenase deficiency, presence of a non- healing wound or fracture, pre-existing sensory or motor neuropathy (grade 2 or more), carcinomatous meningitis, predisposing colonic or small bowel disorders in which symptoms are uncontrolled, prior history of chronic or inflammatory enteropathy, chronic diarrhoea, unresolved bowel obstruction, extensive small intestine resection or presence of a colonic stent. Dipyridamole, allopurinol and sorivudine and its analogues were not permitted, nor were concurrent radiotherapy treatment or evidence of any other disease, metabolic dysfunction or laboratory finding which put the patient at high risk of treatment-related complications. During the trial, safety information was released regarding an increased risk of osteonecrosis of the jaw in patients receiving bevacizumab and concurrent or previous bisphosphonate therapy. Thus a dental examination of such patients prior to study entry was added to the eligibility criteria as a protocol amendment.

The criteria for entry into the trial reflect standard eligibility factors for patients receiving anti-angiogenic therapy. All patients had given written informed consent to participate in the study which was carried out in accordance with standards of Good Clinical practice (GCP)

2.3 Treatment

Eligible patients registered in the study received oxaliplatin chemotherapy with either 5FU or capecitabine and bevacizumab. The oxaliplatin and 5FU regimen shown overleaf in Table 11 is a modified version of the FOLFOX-6 regimen and the standard of care at our institution for patients receiving first line treatment for metastatic colorectal cancer. Treatment within the Travastin-1 trial was based on the NO16966 trial (Saltz, Clarke et al. 2008) which established the use and toxicity profile for the combination of oxaliplatin, 5FU and bevacizumab. In accordance with the N01966 trial, patients who did not wish to have a central venous catheter could receive treatment with the XELOX regimen (Cassidy, Tabernero et al. 2004) comprising oxaliplatin 130mg/m^2 in combination with capecitabine 1000mg/m^2 for 14 days out of 21, every 3 weeks, as shown in Table 12. With this schedule, the dose of bevacizumab was 7.5mg/kg every 3 weeks.

On day 0, two weeks prior to chemotherapy, all patients received a higher dose of bevacizumab (10mg/kg), with the aim of achieving a maximum pharmacodynamic effect on tumour microvasculature and in order to facilitate longitudinal assessment of biomarkers.

Day 0	bevacizumab	10mg/kg	IV infusion 90 mins
Day 14	oxaliplatin	85mg/m^2	IV infusion 2 hours
	L-folinic acid	175mg	IV infusion 2 hours
	5-fluorouracil	400mg/m^2	IV bolus
	5-fluorouracil	2400mg/m^2	IV infusion 46 hours
	bevacizumab	5mg/kg	IV infusion 30-90 mins

Table 11: modified FOLFOX-6 regimen plus bevacizumab

Day 0	bevacizumab	10mg/kg	IV infusion 90 mins
Day 14	oxaliplatin	130mg/m^2	IV infusion 2 hours
	capecitabine	1000mg/m^2	BD per oral 14 days
	bevacizumab	7.5mg/kg	IV infusion 30-90 mins

Table 12: XELOX regimen plus bevacizumab

Patients were monitored for adverse events and severity was recorded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3. Dose modifications were made for grade 3 or 4 toxicities in accordance with the trial protocol. Patients who discontinued oxaliplatin due to toxicity continued with 5-fluorouracil (5FU) chemotherapy in combination with bevacizumab. Patients continued on treatment with chemotherapy and bevacizumab every 2 weeks until disease progression, withdrawal of patient consent or unacceptable toxicity. Oxaliplatin chemotherapy was discontinued after a maximum of 12 cycles in patients who received FOLFOX and 8 cycles in patients who received CAPOX.

Radiological response assessment was evaluated using standard anatomical CT assessment at baseline (within 28 days of treatment with bevacizumab) and every 12 weeks according to RECIST 1.0 (Therasse, Arbuck et al. 2000). This version of RECIST was still in use at our institution when the Travastin-1 trial opened to recruitment in 2009 and was continued for the duration of the trial to maintain consistency.

2.4 Biomarkers

2.4.1 DCE-MRI acquisition

All DCE-MRI scans were acquired at the Wolfson Molecular Imaging Centre, Manchester in the presence of a research radiographer familiar with the study protocol.

Patients had two pre-treatment MRI scans within the 14 days before receipt of the first dose of bevacizumab. Follow-up scans were then carried out according to the schedule. The tumour of interest(s), a minimum of 3cm in diameter, was selected using recent standard anatomical CT images. In selected cases this required consultation with a radiologist. The same tumour of interest(s) was used for all subsequent scans for an individual patient. For an individual patient, where more than one tumour of interest measuring 3cm in diameter was visible in the MR field of view, these data could be included in the analyses. To maintain consistency, patients were scanned at the same time of day whenever possible with contrast injected into the same vein, using an identically sized cannula on each occasion.

Data were acquired on a 1.5T Philips Intera whole body imaging system located at the Wolfson Molecular Imaging Centre, Manchester. Anatomical images were acquired using a Torso XL coil. A coronal T1 survey scan was used to localize the region of interest (ROI). Anatomical images were acquired to define the ROI, these included:

1) Trans-axial pre-contrast T1 image (Fast field echo (FFE): TR 10ms, TE 4.6 ms, flip angle (FA) 15**°,** matrix 256 x 256, FOV 375mm)

2) T2-weighted image (short spin echo-EPI with spectral inversion recovery fat suppression, TR 541ms, TE 80ms, FA 90**°,** matrix 256 x 256, FOV 375mm**).**

The DCE-MRI sequence and baseline T1 measurement were acquired using the scanner whole body coil (Q body coil) for transmission and reception. The baseline T1 mapping measurement consisted of 3 axial spoiled fast field echo (T_1-FFE) sequences. A variable flip angle acquisition was used to calculate baseline T1 measurement using different flip angles (2°, 10°, 20°) and four signal averages. The geometry for both the baseline T1 measurements and the dynamic series was consistent (25 slices, FOV (375 mm × 375 mm), matrix size (128 × 128), TR (4.0 ms), and TE (0.82 ms). Slice thickness was 4 mm for small target lesions or 8 mm for larger lesions (defined as more than 10cm) giving cranio-caudal coverage of 100 mm or 200 mm, respectively. The DCE-MRI series consisted of 75 consecutively-acquired axial volumes (FA 20°, 1 signal average, temporal resolution of 4.97 s). This resulted in a total duration of 6 minutes 13 seconds for the DCE-MRI acquisition.

Gadoterate Meglumine(0.1mmol/kg; Dotarem®, Guerbet UK) was injected intravenously (IV) by power injector at the time of the sixth dynamic acquisition at 0.2ml/kg, followed by a 20ml saline flush at a set rate of 3ml/sec. This was followed by acquisition of a post contrast T1-weighted image, identical to the pre contrast T1 protocol. A free- breathing technique was used throughout all acquisitions and post processing registration was applied in cases where significant motion was observed during quality control.

2.4.1.1 DCE-MRI data analysis

Data analysis was performed in conjunction with the Quantitative Biomedical Imaging Lab (QBI Lab, University of Manchester). The DCE-MRI data were analysed according to GCP standards using in house software Manchester Dynamic Imaging (MADYM).

Volumes of interest (VOIs) were manually delineated in 3D on the series of MR scans by an experienced radiographer on co–registered high resolution T1- and T2-weighted images.

Files were created which included only voxels from the tumour ROI; no voxels within this tumour volume were rejected. This gave a measurement of whole tumour volume (WTV) for each lesion.

An arterial input function was manually identified from the largest vessel in the field of view, in most cases the aorta. In circumstances where it was not possible to obtain a calculated input function, due to small vessel diameter, a population derived input function was used (Parker, Roberts et al. 2006).

Concentration time course data were created for each voxel and the initial area under the gadolinium contrast concentration curve at 60s (IAUC-60) was calculated, providing a semi-quantitative measure of tumour perfusion (Evelhoch 1999). Enhancing voxels from tumour VOIs were included in the pharmacokinetic analysis if they demonstrated uptake of contrast. Contrast uptake was defined as IAUC-60 >0mmol/s. The tumour Enhancing Fraction (EF) was calculated according to the following equation: (enhancing tumour volume/ total tumour volume) x 100. This gave an approximation of the proportion of the tumour receiving perfusion (Jayson, Parker et al. 2005; Jackson, O'Connor et al. 2007).

Pharmacokinetic analysis of DCE-MRI was performed by fitting concentration time course data from enhancing voxels to the extended Tofts and Kermode pharmacokinetic model (Tofts, Brix et al. 1999) using in house software (Manchester Dynamic Modelling). Baseline T1 values were used to transform signal intensity time course curves to contrast concentration curves.

Median values for volume transfer coefficient (K^{trans}), extracellular extra-vascular space (Ve) and blood plasma volume (*Vp)* were determined from the enhancing component of each tumour VOI.

Reproducibility values were calculated for each parameter from the two baseline MR scans. The number of lesions used to calculate the reproducibility of each parameter are given in Table 17 (Chapter 4), and varied between 111 and 119 paired datasets depending on parameter.

2.4.2 DWI acquisition

Diffusion sequences were acquired following pre-contrast T1 and T2-weighted images. The diffusion weighted imaging (DWI) protocol used single shot EPI images TR (3416ms) and TE (90ms) with transverse, trans-axial slices (thickness 4mm). Three gradient directions were applied: *modulus*- the direction at 90 degrees to the phase direction, *phase*- the anterior-posterior (AP) direction and *slice*- the foot-head direction.

Four b values were acquired (0,150, 500 and 800) to allow fitting to an exponential curve. A high b value of 800 was based on previous studies within the group which had demonstrated sufficient signal, as it was felt that the signal above 800 would be unreliable. A b value of 150 was selected as this would be above the level where capillary flow effects would be expected. A b value of 500 was selected as this was roughly half way between the other two b values selected and would aid fitting of the exponential curve. As the inclusion of b values of 0 in the fitting of the exponential curve might potentially result in sensitivity to changes in capillary perfusion which could lead to over-estimation of the ADC, b values of 0 were excluded from the fitting.

A separate ROI was drawn for the DWI images and mask files created containing only voxels from the tumour ROI. Apparent diffusion coefficient (ADC) maps were constructed using in-house software (Manchester Dynamic Modeling).

2.4.3 Serological markers of angiogenesis

A 6ml blood sample was obtained using a standard EDTA blood collection tube and plasma was prepared by centrifugation at 2000g for 5 minutes within 30 minutes of collection. Plasma was divided into 500µl aliquots prior to storage at -80°C.

Plasma samples were analysed by a research scientist in the Cancer Research UK Clinical and Experimental Pharmacology Good Clinical Practice laboratories (GCP-L), Paterson Institute for Cancer Research, University of Manchester. A multiplex ELISA method was used (SearchLight Plus multiplex ELISA platform, Aushon BioSystems, Boston, US) which had been previously validated in-house (Backen, Cummings et al. 2009). Eighteen different assays were arranged into 6 different plexes as follows: 2 six-plex ELISAs containing assays for Ang2, FGFb, HGF, PDGFbb, VEGFA and VEGFC and IL6, IL8, KGF, PlGF, VEGFR1 and VEGFR2, 2 two-plex ELISAs containing assays for Ang1 and Tie2 and E Selectin and VCAM1 and 2 single-plex ELISAs each containing an assay for SDF1b and VEGFD.

The M65 ELISA, Peviva (Bromma, Sweden), was used for quantification of total cell death (due to apoptosis and necrosis) by measurement of total soluble circulating levels of cytokeratin 18 (both caspase cleaved and intact forms) after release from dying epithelial cells. This has been previously validated in our laboratory (Greystoke, Cummings et al. 2008).

Time constraints on the data analysis and writing of this thesis necessitated the reporting of only a sub group of the analysed circulating biomarker data: VEGF-A, VEGF-D, Ang-1, Ang-2, Tie-2, E-selectin, VEGFR-2 and HGF. These were selected prior to any data analysis involving outcome data.

2.5 Statistical considerations

2.5.1 Statistical methodology for Travastin-1 trial design

The statistics department at the Christie NHS Foundation Trust Clinical Trials Unit advised on the statistical design and power calculations for the study. The primary end point of the study was progression free survival which was calculated as the interval between the date of consent and the date of radiological progression as defined above or, death from disease or unknown cause. The study was also designed to evaluate clinical outcome measures of response rate and overall survival.

Based on data accrued one year after the last patient had entered the study, analysis of the biomarker data was designed to have an 80% power to detect biomarker stratified groups with a difference of at least 20% in progression free survival, using a 2-tailed tested and 5% significance levels. Once all patients had progressed, the study would have 95% power to detect a correlation between a biomarker and progression free survival of 0.42 or greater, using a 2-tailed test and 5% significance levels.

2.5.1.1 Statistical methods for data analysis

Results were analysed using Prism (Graph Pad Software Inc., CA, USA) and SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). All p-value determinations were two sided and significance was defined as a $p<0.05$.

The Kaplan-Meier method was used to obtain estimates of median progression free survival and overall survival for patients on the trial. A univariate cox regression analysis was used to investigate potential prognostic factors in relation to outcome and a log- rank test was used to compare estimates of progression free survival for significant factors. Continuous variables were dichotomised into high and low values according to the median value of the variable.

An independent t-test or Mann-Whitney U test was used to compare groups, depending on whether data conformed to a Gaussian or non-Gaussian distribution.

The association between median levels of biomarker and best radiological response measured according to RECIST criteria was investigated using the Kruskal-Wallis test. Best radiological response was categorised into 3 groups according to definitions from RECIST version 1.0 (Therasse, Arbuck et al. 2000): Complete or partial response (group 1), stable disease (group 2) or progressive disease (group 3). A chi-squared test was used to compare groups of categorical variables. Correlations between continuous variables were assessed using Pearson's test if at least one of the variables conformed to a Gaussian distribution or Kendall's test if this condition was not satisfied.

For the imaging data, values from individual tumours in each patient were averaged to obtain a single value for each parameter per patient, in order to facilitate statistical comparison between patient groups.

2.5.1.2 Multivariate analysis

The most significant prognostic factors were selected from the results of univariate analyses of clinical variables, DCE-MRI parameters and angiocytokines for inclusion in a multivariate cox regression model for PFS. Where variables were very highly correlated, such as K^{trans} and IAUC (Pearson's correlation 0.83, $p<0.0001$), the most biologically relevant parameter was selected; in this case, K^{trans}. The variables selected were: serum LDH, platelet count, CEA (dichotomised according to the median value, 47ng/mL), ECOG performance status (0 versus 1 or 2), K^{trans} (dichotomised according to the median value of $0.159min^{-1}$), HGF (dichotomised according to the median value of 415pg/mL), VEGFR-2 (dichotomised according to the median value of 11731pg/mL) and VEGF-A.

These variables were entered into a multivariate cox regression model using a backward conditional method.

2.5.2 Statistical methodology for assessing reproducibility

Inter-visit reproducibility for each parameter was assessed using the Bland-Altman technique (Bland and Altman 1986). Data from individual tumours in each patient were averaged to obtain a single value for each parameter per patient. An average baseline value for each parameter was calculated from two pre-treatment visits, for each tumour lesion. Transformed variables were used for all subsequent statistical analysis where this was indicated by previous reproducibility assessments.

Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk test. Correlations were tested using Kendall's test or Spearman's test, depending on whether data were consistent with a Gaussian distribution. In cases where the parameter distribution was non-Gaussian or, where there was evidence of a relationship between the difference in parameter and parameter mean; data were transformed using a log normal transformation. Distributions were then retested according to the preceding method. If log natural transformation did not improve parameter distribution, advice regarding further transformation was sought from a biomedical statistician based at the University of Manchester with knowledge of the project. The within patient co-efficient of variation (CV), was calculated according to previously described methods used by our group (Galbraith, Lodge et al. 2002).

2.5.3 Methods for investigating heterogeneity in patients with colorectal liver metastases

All patients underwent two pre-treatment DCE-MRI scans within a one-week period (median 4 days; range 2-7 days) prior to treatment. To reduce heterogeneity effects related to variations in tumour microenvironment, only patients with liver metastases were included in this analysis.

Histograms were plotted for median values of each variable (K^{trans}, V_e, V_p, EF, ADC) from the first baseline visit. Repeated measurements from the baseline scans were used to construct Bland-Altman plots to establish measurement reproducibility (Bland and Altman 1986). Bland-Altman plots were then used to construct empirical models of measurement accuracy for parameters ADC, K^{trans}, v_e and v_p in the form $x \pm kx^{\frac{n}{2}}$ (for integer n), while the EF value was assumed to be distributed as a binomial random variable. The resultant empirical error models for each of these summary parameters ($f(x)$) was used to derive a corresponding non-linear mapping function $y(x)$ allowing transformation of measured median values to an approximate Gaussian function with homogenous measurement errors.

$$y(x) \propto \int_{f(x)}^{1} dx$$

The theory of error propagation (a first order approximation), can be used to show that $y(x)$ will have approximate homogenous measurement error. Variances around the mean for the upper and lower data ranges were then found to be consistent (within statistical error).

A chi-squared test (with 5 degrees of freedom) for the difference between tumours i and j was then defined as the sum of squares of the difference between changes in each derived DCE MRI variable i divided the reproducibility variance:

$$x_{jk}^2 = \sum_i^5 \frac{(y_{ij} - y_{ik})^2}{\sigma_{yi}^2}$$

The quantity $D_{jk} = \sqrt{x_{jk}^2}/5$ would be expected to behave as a linear Euclidian distance of measurable statistical difference. A mean value of 1 will be generated for data j and k, which differs only due to the presence of the modelled level of measurement error (σ_y). The statistical distance D was therefore constructed for differences between tumours of each subject, and also for differences between tumours from different subjects.

Chapter 3: Clinical Results

3.1 Patient Characteristics

From November 2009 to October 2012, 76 patients with metastatic colorectal cancer were enrolled in the Travastin-1 trial.

Seventy patients received treatment within the trial. Five patients were found to be ineligible during the screening period and prior to receiving any treatment. The reasons for trial ineligibility included: uncontrolled hypertension (n=1), recent surgical procedure (n=1), a recently diagnosed venous thromboembolism (n=1), a recent history of rectal bleeding (n=1) and histological diagnosis of neuroendocrine tumour (n=1). A further patient withdrew consent from the study and received chemotherapy out with the trial.

The six patients who did not receive any treatment were not included in any further analysis. A CONSORT diagram for the trial is shown in Figure 1.

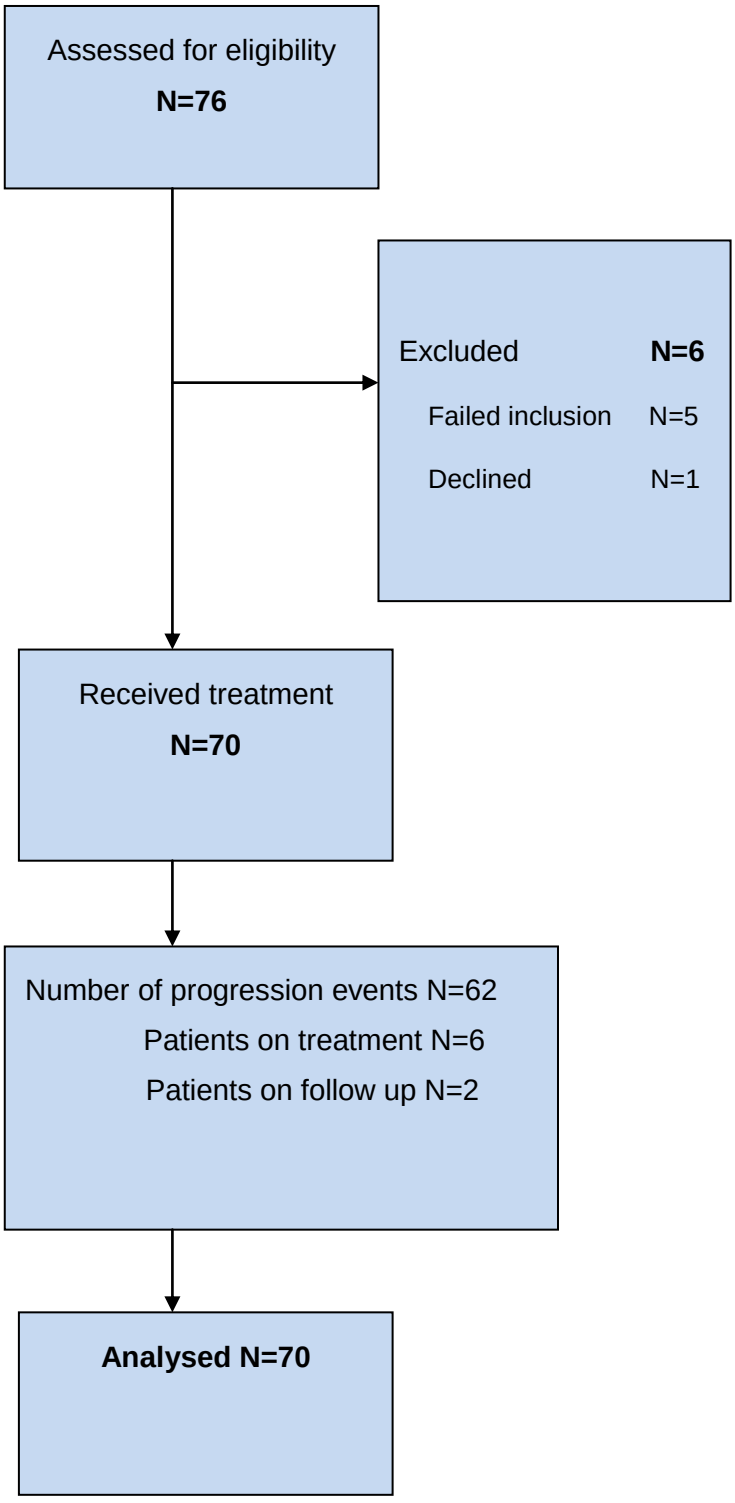

Figure 1: CONSORT diagram for Travastin-1 trial

The characteristics of the patients treated in the Travastin-1 study are shown in Table 13. The male to female ratio was (3:2) and the median age for the group was 63 years (range 29-77 years). The majority of patients (96%) had a European Clinical Oncology Group (ECOG) Performance score (PS) of 0 or 1. Metastatic involvement of 2 or more organs was common (56% of patients). Only 22 patients (31%) had undergone resection of their primary tumour. The tumour grade was moderately differentiated in the majority of patients; 46/70 patients (66%). Since one of the main conditions for trial entry was the presence of disease >3cm for imaging purposes, the trial was biased towards patients with large disease volume. This was reflected in the high median pre-treatment plasma CEA and LDH concentrations.

During the period that patients were recruited to our study, testing for activating mutations in the Kirsten RAS (KRAS) gene was not routinely performed at our institution. Consequently, the KRAS mutational status was unknown for the majority of the patients participating in our trial.

Patient Characteristics		N (%)
Sex-n (%)	male female TOTAL	41 (59) 29 (41) 70
Age, years	median range	63 (29-77)
ECOG performance status (PS)	0 1 2	39 (56) 28 (40) 3 (4)
Laboratory investigations	Platelets (median; range) LDH (median; range) CEA (median; range)	373 (172-931) 475 (157-9335) 47 (<3-16,485)
Disease sites-N (%)	Liver Lung Nodes Bone Other	63 (90) 25 (36) 24 (34) 1 (1) 11 (16)
No. of metastatic sites-N (%)	1 2 3 or more	31 (45) 26 (37) 13 (18)
Tumour grade-N (%)	Well differentiated Moderately differentiated Poorly differentiated unknown	7 (10) 46 (66) 9 (13) 8 (11)
Primary tumour resected N (%)	Yes No	22 (32) 48 (68)

Table 13: Baseline characteristics of patients on the Travastin-1 study.

Data are presented as N (%) unless otherwise specified.

3.2 Treatment administration and toxicity

As of August 5th 2013, the median number of administered treatment cycles was 14 with a range of 1 to 61. Eight patients remained on study at this time of whom, six were still receiving treatment. Treatment was generally well tolerated and toxicities were consistent with those reported elsewhere in the literature (Saltz, Clarke et al. 2008). Adverse events were mostly grade 1 or 2; the most prevalent being nausea, peripheral neuropathy, fatigue, epistaxis and neutropenia. Fifteen patients discontinued therapy due to adverse events (AEs): in 12 of these patients the AEs were grade 3 or 4. The events of special relevance to bevacizumab that led to study discontinuation are shown in Table 14 overleaf.

AEs leading to discontinuation of treatment (Total study population 70 patients)	N (%)
Any	15 (21)
Grade 3/4	12 (17)
Event of relevance to bevacizumab	12 (17)
Venous thromboembolism	3 (4)
Bleeding	3 (4)
Arterial thromboembolic	3 (4)
Fistula/ intra-abdominal abscess	2 (3)
Perforation	1 (1)
Proteinuria	1 (1)
Hypertension	0
Wound healing complication	0

Table 14: Events of relevance to bevacizumab leading to discontinuation of treatment.

3.3 Treatment discontinuation

At the time of data capture (August 5th, 2013), 62 patients had discontinued therapy and had reached the primary end point (PFS). The median follow up time for the eight patients who had not reached the primary end point was 296 days (171-1143 days). Even if some patients with a long progression free survival were excluded from this analyses because they had not reached the primary end point, the median progression free survival should be unaffected by a few potential outliers.

The most common reason for discontinuation of treatment was disease progression, which accounted for 37 of the 62 (60%) patients. Fifteen patients (15/62) (21%) were withdrawn from study as a result of toxicity as described above.

Patients on maintenance treatment were allowed to interrupt treatment with bevacizumab for no more than 6 weeks (3 cycles) before being required to come off study. Six patients (6/62) (10%) who initially had inoperable disease were referred for liver resection following treatment, although one patient subsequently declined surgery.

Bowel obstruction, secondary to a primary colorectal cancer which remained in situ, occurred in two patients (2/62). A patient with a sigmoid cancer developed sub-acute bowel obstruction after two cycles of treatment and required insertion of a colonic stent. As the presence of a colonic stent was one of the exclusion criteria for this trial, the patient left the study. This patient continued on chemotherapy and subsequently had a complete response that was maintained at the time of data analysis. A further patient developed sub-acute bowel obstruction due to a primary in the ascending colon and required a de-functioning procedure. This patient resumed chemotherapy on the trial following the procedure but treatment with bevacizumab was discontinued.

Two patients (2/62) withdrew study consent during maintenance chemotherapy with 5-FU and bevacizumab as they wished to have treatment at their local hospital.

3.4 Efficacy and outcome

Radiological response to treatment was determined according to RECIST 1.0 criteria (Therasse, Arbuck et al. 2000) (Table 15). The overall objective response rate for patients on our trial was 31/70 (44%). This was defined as the number of patients who obtained either a complete or partial response. Eight patients (11.5%) had evidence of disease progression by the first radiological response assessment at 12 weeks.

The median PFS interval, based on events in 62 of 70 patients, with remaining patients censored on August 5th, 2013, was 9.3 months (95% CI, 8.5 to 11.3 months), Figure 2.

Best objective response category (Total N=70)	N (%)
Complete response	1 (1)
Partial response	30 (43)
Stable disease	29 (41.5)
Progressive disease	8 (11.5)
Unevaluable	2 (3)
Overall objective response rate	**31 (44)**

Table 15: Best objective response based on RECIST

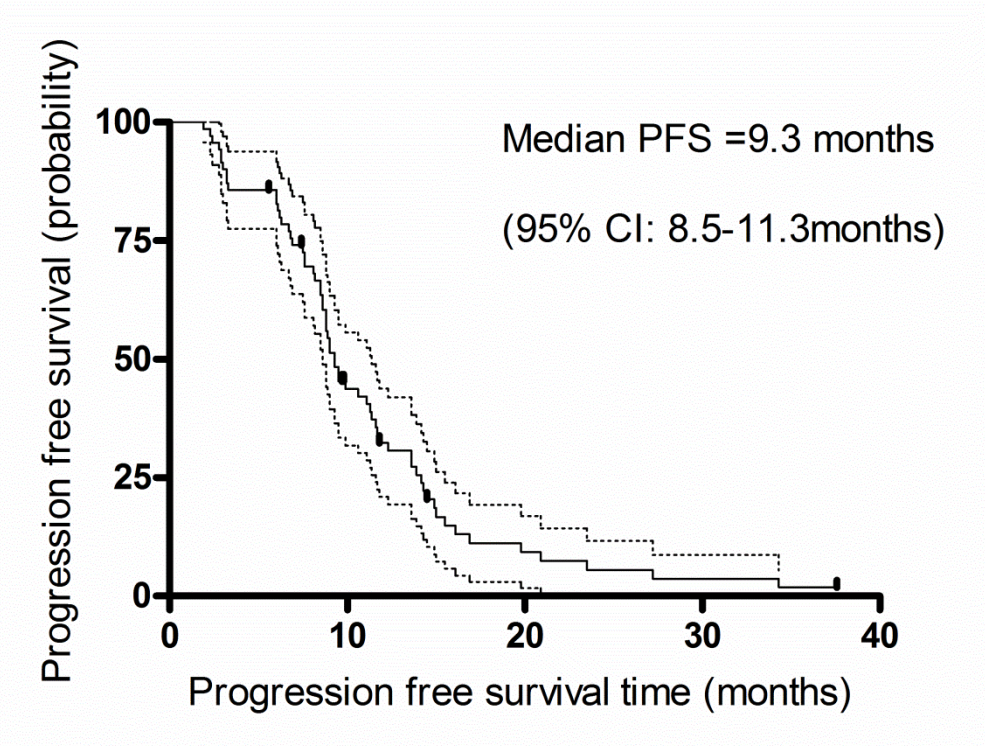

Figure 2: Kaplan-Meier estimate for progression free survival.

Progression free survival (PFS) is shown for all patients with 8 censored patients indicated by ticks. Dotted lines indicate 95% confidence interval for estimates.

The outcome of patients according to known prognostic factors in colorectal cancer was investigated using a univariate cox regression model. The significance of each clinical variable with progression free survival (PFS) is shown in Table 16.

The outcome of patients according to prognostic factors identified by univariate analysis is demonstrated in Figure 3(A-D). A log-rank test was used to compare Kaplan-Meier estimates of progression free survival (PFS). There was a significant difference in the median PFS according to ECOG performance status: 50.9 weeks (PS=0) v 37.1 weeks (PS=1) v 27. 3 weeks (PS=2), $p<0.0001$ (Figure 3A).

The median value for CEA for patients in this trial was 47µg/L (range: 3-16,485µg/L). Patients dichotomised according to the median value of CEA showed no difference in median PFS (40.4 v 40.3 weeks, HR 0.58 (95% CI, 0.32-0.93), p=0.03), although the survival curves diverged significantly beyond the median position (Figure 5B). Patients with a low LDH (less than the median value of 475) had a better progression free interval than those with a high LDH: 49 v 37 weeks (HR 0.55 (95% CI, 0.33-0.93) p=0.0147) (Figure 3C). Although a lower platelet count (less than $400x10^9$/L) was associated with a better outcome: 50.3 v 38 weeks, this difference was not significant (HR 0.07 (0.4- 1.1), p=0.1) (Figure 3D).

Variable	Hazard Ratio (95% CI)	Significance on univariate analysis
Age (≤65years or >65years)	1.2 (0.7-2.0)	p=0.52
Gender (M v F)	0.8 (0.5-1.3)	p=0.35
Number of metastatic sites	1.1 (0.6-2.1)	p=0.8
Grade (3 v 2)	2.8 (0.9-9.0)	p=0.8
LDH	1.7(1.2-2.4)	p=0.005*****
Platelets	1.9 (1.0-3.8)	p=0.07
CEA (“low” versus “high”)**	0.6 (0.3-1.0)	p=0.04*****
ECOG (0 v 1/ 2)	0.5 (0.3-0.8)	p=0.005*****

Table 16: Univariate analysis of clinical prognostic factors in metastatic colorectal cancer.

Log-normal values of LDH and platelets were used. Significance assessed by univariate cox regression analysis against progression free survival. HR>1.0 indicates a higher risk of relapse for high v low values of the biomarker.

* indicates significance at the 0.05 level

** indicates where data were dichotomised according to the median value

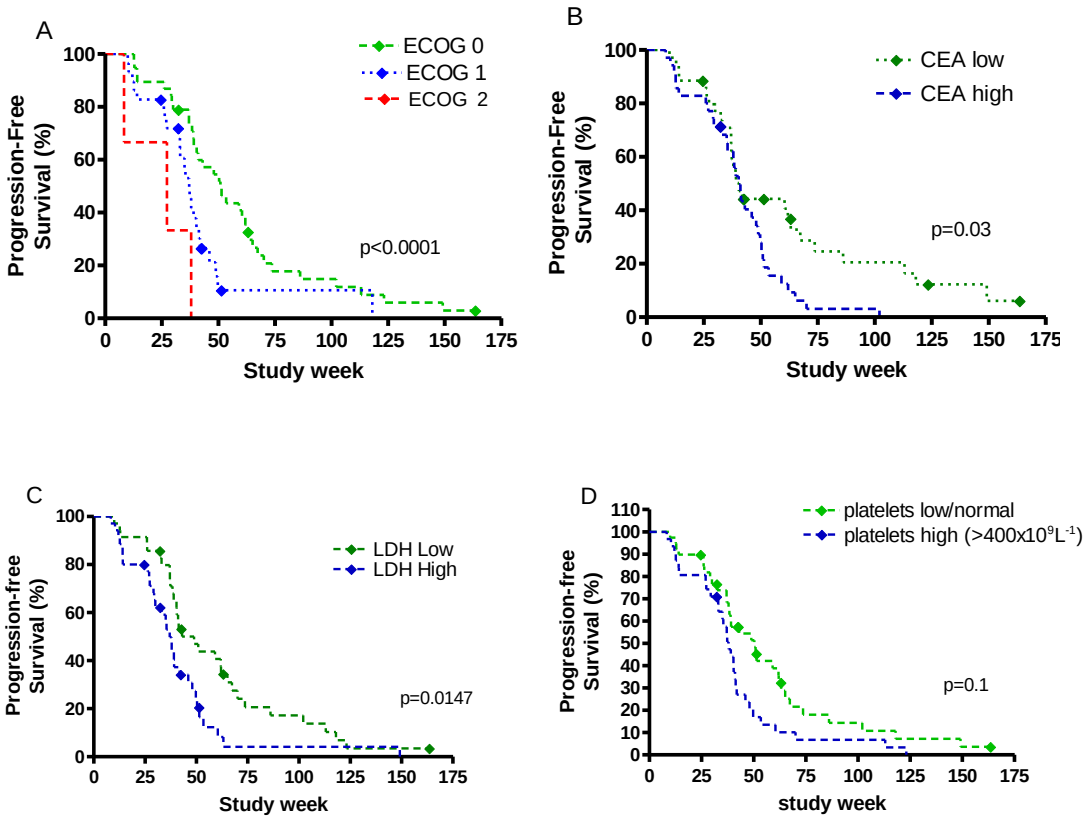

Figure 3: Progression free survival by prognostic factor.

Figures represent Kaplan-Meier estimates of progression free survival (PFS) following treatment with bevacizumab and chemotherapy in colorectal cancer related to the following prognostic factors**:**

A. ECOG performance status.

B. CEA above and below the median value of 47µg/L.

C. LDH above and below the median value of 475U/L.

D. Platelet count above and below $400x10^{9}L^{-1}$.

Associations between these clinical prognostic factors were explored. Those that were associated with performance status were platelet count and LDH. In each case higher values were associated with a worse performance status. There was a significant difference in median values of LDH between patients who were PS 0 compared with those who were PS 1 or 2 (454 v 573, p=0.02, Mann-Whitney U test). The relationship between platelet count and performance status is shown in Figure 4. The median platelet count for patients who were PS 0 was 321 $x10^9$/L compared with 424 x 10^9/L for patients who were PS 1 or 2 (p=0.011, independent t-test).

Pre-treatment levels of serum LDH were dichotomised according to median values and compared across radiological response categories. However there was no difference in radiological response depending on whether patients had a high or low levels of LDH, Chi-squared test (p=0.8).

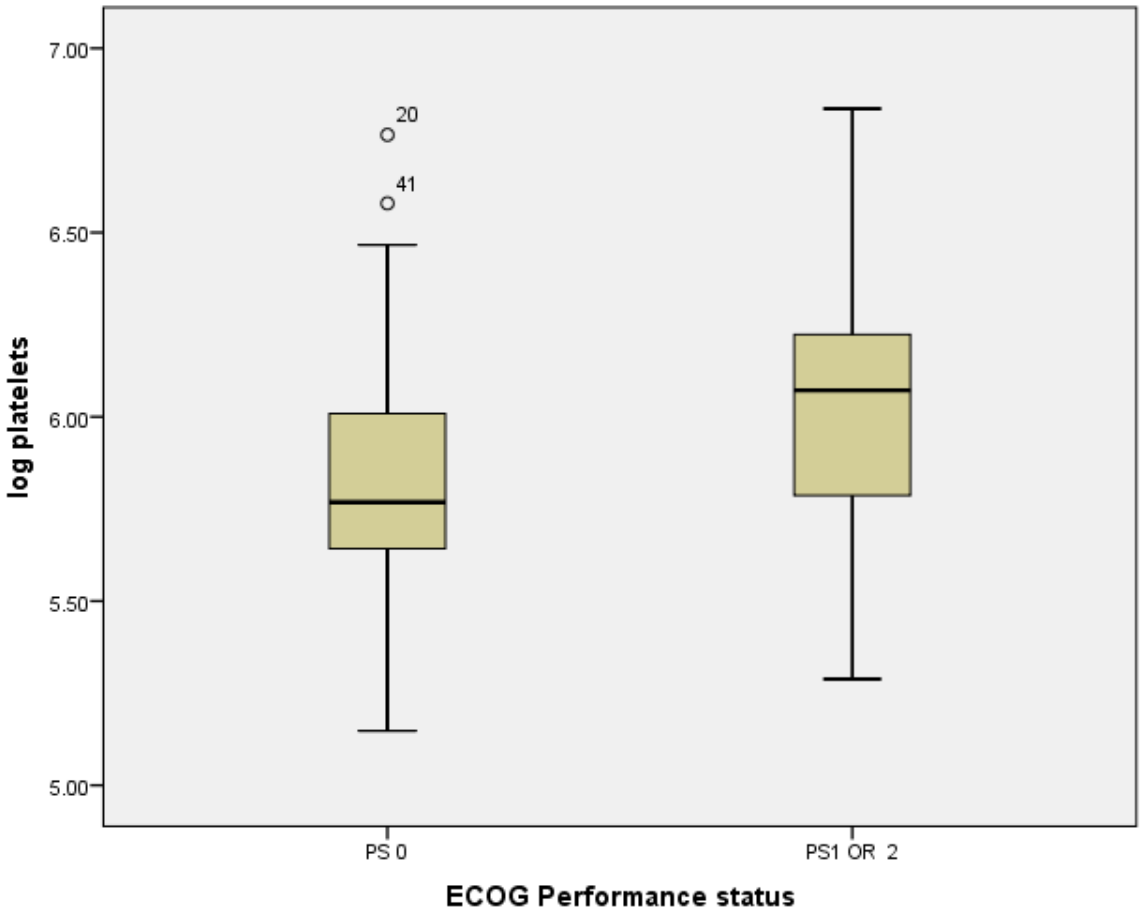

Figure 4: Median values of platelet count according to performance status.

Box plots represent median (range) of values of platelet count according to performance status. Two outliers are represented by open circles and labelled according to patient ID number. These plots illustrate that patients with an ECOG performance score of 1 or 2 had a higher median platelet count (424 x 10^9/L) than patients with an ECOG performance score of 0 (321 x 10^9/L). This difference was statistically significant according to an independent t-test (p=0.011). A log normal value of platelet count was used and back transformation of median values was used to obtain standard measurements.

3.4.1 Overall survival

At the time of data cut off, 42 death events had occurred. In 39 subjects, death was due to progression of the disease. In the remaining three patients the cause of death was possibly related to treatment toxicity. A 60 year old woman developed a fatal perforation of the gall bladder ten weeks after completing 29 cycles of bevacizumab, which had been stopped due to the development of progressive disease. A 64 year old man developed a perforation of the sigmoid colon 19 weeks after completing 11 cycles of bevacizumab, oxaliplatin and 5FU chemotherapy. Following chemotherapy, this latter patient had undergone further chemo-radiotherapy of a sigmoid primary and was awaiting a planned surgical resection of the primary cancer and synchronous liver metastases. A 58 year old man developed fatal duodenal ulceration and bleeding after 25 cycles of bevacizumab.

Kaplan-Meier survival estimates for the total trial population are shown in Figure 5.

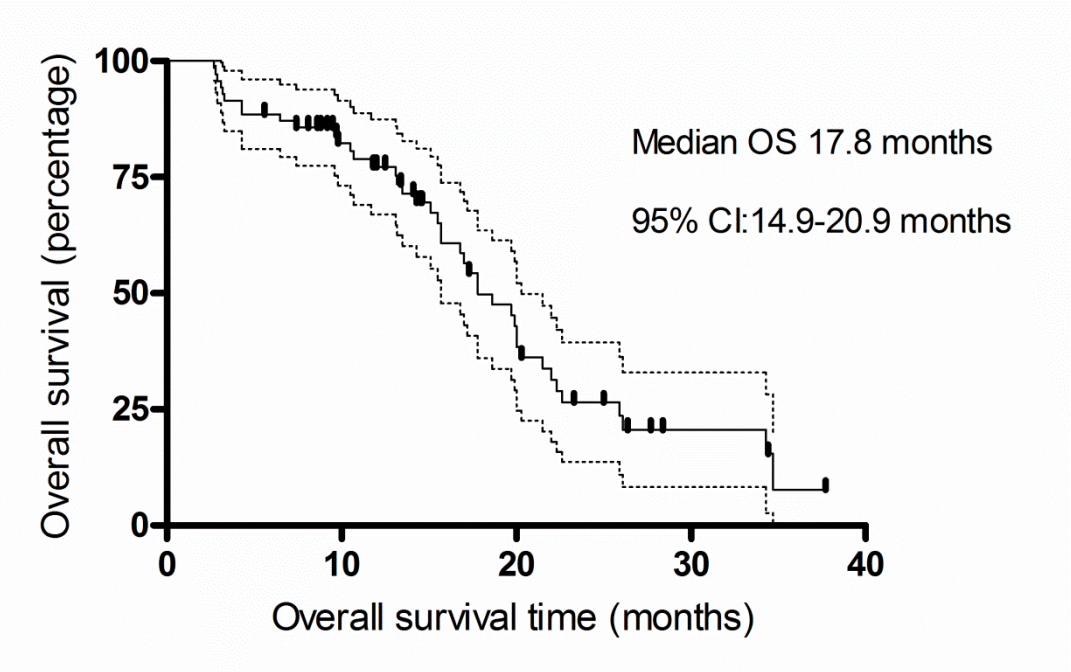

Figure 5: Overall survival curves for the Travastin-1 study population.

The Kaplan-Meier estimate of overall survival based on 42/70 events. The 28 censored patients are indicated by ticks. Dotted lines indicate 95% confidence interval for estimates.

3.5 Discussion of clinical results

The characteristics of our study population are consistent with those reported in two comparable large randomised phase 3 trials investigating bevacizumab in combination with chemotherapy for the first line treatment of metastatic colorectal cancer (Hurwitz, Fehrenbacher et al. 2004; Saltz, Clarke et al. 2008). Significantly more men than women were represented in our trial population (59% vs 41%). However, this is in keeping with the known epidemiology of colorectal cancer, and the figures are consistent with gender distributions in these other trials. The median age at diagnosis for patients with metastatic colorectal cancer is 70 years (Brenner, Kloor et al. 2013) which is higher than the median age of 63 years found in this trial population. However this is comparable with the median age of patients participating in the N016966 study (Saltz, Clarke et al. 2008) and the AVF2107 trial (Hurwitz, Fehrenbacher et al. 2004) (60 years and 59 years, respectively).

Performance status is known to be an important prognostic factor in colorectal cancer (Kemeny and Braun 1983; Kohne, Cunningham et al. 2002). Researchers who analysed 6286 patients with metastatic colorectal cancer from nine trials (Sargent, Kohne et al. 2009) found that less than 8% of patients had a ECOG PS of 2. Furthermore outcomes of PS 2 patients were significantly worse than those for patients with PS 0 or 1. In patients where poor performance status was due to disease there was still a benefit from combination therapy although this was reduced when compared to patients with a better performance status. The role of biological therapies in poor performance status patients remains poorly defined as few trials involving biological agents include such patients. In the N016966 study and the AVF2107 trial there were less than 1% of patients with PS 2. These data are comparable to the trial population presented here, which included three patients (4%) with PS 2.

Researchers investigating prognostic factors in colorectal cancer have used different methods of stratifying patients according to CEA values. Kohne and colleagues used multiple cut points (30, 100, 500, 1000 µg/l) in large data set which included 3825 patients from 19 clinic trials (Kohne, Cunningham et al. 2002). Using this method, CEA did not emerge as a significant prognostic factor in multivariate analysis. Other researchers looked at cut points above 5µg/l, upper limit of normal (ULN) or 50µg/L (10xULN)(Webb, Scott-Mackie et al. 1995). Values above either cut point were significantly associated with a poorer survival on multivariate analysis. Given the wide range of CEA values for patients in this trial and the fact that only 9 patients had values on or below ULN, the median cut point of 47µg/L can be justified. Dividing the CEA values into the multiple cut points used by Kohne would not have been appropriate for a study of this size.

Evidence of an association between performance status, LDH levels and platelet count, supports the hypothesis that poor performance was related to disease burden in our population. The relationship between LDH and disease burden has been observed in other trials of metastatic colorectal cancer patients (Koukourakis, Giatromanolaki et al. 2011) but this may be the first report of a relationship between platelet count and performance status in metastatic colorectal cancer. However, platelet count is recognized to be an adverse prognostic factor in colorectal cancer (Kohne, Cunningham et al. 2002) and the COIN study investigators reported a poorer prognosis for a subgroup of patients with a platelet count above 400 x 10^9/L who received intermittent chemotherapy compared with continuous chemotherapy (Adams, Meade et al. 2011). Consistent with these results from the COIN study, our patients, all of whom were on continuous treatment until the progression of their cancer, showed no difference in outcome according to a pre-treatment platelet count dichotomised above or below 400 x 10^9/L.

Response rate is often a secondary end point of large randomised trials in colorectal cancer and consequently these data are not always published for individual trials. The TREE study reported disease progression in 6/71 (8.4%) patients receiving FOLFOX plus bevacizumab and 8/72 (11.1%) patients receiving CAPOX plus bevacizumab. These findings are consistent with the rate of disease progression of 9% of patients presented here. A best overall response rate for patients treated with bevacizumab of 39% (95% CI 37%-42%) was recently reported in a pooled analysis of seven trials carried out by Hurwitz and colleagues (Hurwitz, Tebbutt et al. 2013) and is consistent with the observation of an overall response rate of 44%. Although serum LDH has been proposed as a marker of angiogenesis in colorectal cancer (Scartozzi, Giampieri et al. 2012), no association was demonstrated in this study between serum LDH and response rate.

The resection rate in this study of 10% was consistent with the 8.4% resection rate reported in the N016966 study (Saltz, Clarke et al. 2008). Similarly, a secondary resection rate of 11.8% was described in the First Beat expanded access trial of bevacizumab (Van Cutsem, Rivera et al. 2009).

Median progression-free survival in this analysis of 9.3 months is comparable to larger published studies involving bevacizumab and combination chemotherapy containing oxaliplatin (Hochster, Hart et al. 2008; Saltz, Clarke et al. 2008). The median overall survival was 17.8 months, which is less than the median overall survival of 21.3 months reported in the Saltz study. More recently, a pooled analysis from Hurwitz and colleagues has demonstrated an overall survival of 19.8 months following treatment with bevacizumab in the first line setting (Hurwitz, Tebbutt et al. 2013).

It is likely that the shorter survival shown in the current study reflects the high disease burden in this trial population, a direct consequence of trial inclusion criteria which required patients to have at least one lesion measuring 3cm or more.

In conclusion, the patient population and outcomes for this trial are consistent with those reported in the literature for patients with metastatic colorectal cancer receiving first line combination chemotherapy and bevacizumab. Although overall survival was slightly less than might be expected this is potentially attributable to the fact that the patients in this study were preselected for a high disease burden with disease measuring 3cm or more, albeit with a good performance status.

Subsequent chapters of this thesis will investigate DCE-MRI imaging parameters and circulating biomarkers in this trial population. In particular, attention will be directed towards ascertaining whether certain biomarkers may be useful in predicting which patients are more likely to benefit from treatment with bevacizumab.

Chapter 4: DCE-MRI to assess microvascular characteristics of colorectal metastases.

4.1 Introduction

This chapter concerns the assessment of microvascular characteristics of colorectal metastases prior to any therapy, using the biomarker methods and the patients described in Chapter 2. The aim was to explore the potential of imaging parameters derived from DCE-MRI and Diffusion-Weighted MRI (DWI) and angiocytokines as prognostic factors in patients treated with bevacizumab and modified FOLFOX-6 chemotherapy. The hypotheses for this chapter are that circulating angiocytokines, DCE-MRI and DWI parameters measured prior to therapy with chemotherapy and bevacizumab are associated with clinical outcome measures such as response rate and progression free survival (PFS).

The aims of this chapter are to:

1. Establish the reproducibility of selected angiocytokines, DCE-MRI and DWI parameters in patients with metastatic colorectal cancer.

2. Investigate the association between DCE-MRI and DWI parameters and clinical prognostic factors in colorectal cancer patients.

3. Investigate the association between angiocytokines, DCE-MRI and DWI parameters and clinical outcome in colorectal patients.

4. Investigate any correlations between circulating and imaging biomarkers pre-treatment

5. Investigate the utility of imaging biomarkers in the assessment of intra- and inter subject heterogeneity

4.2 Baseline DCE-MRI and DWI results

4.2.1 Parameter reproducibility

From a total of seventy patients enrolled in the Travastin-1 trial, sixty-four patients had paired scans suitable for the assessment of DCE-MRI parameter reproducibility. Paired baseline scans for six patients were unavailable or excluded from the analysis for the following reasons: excessive motion which precluded further analysis of at least one baseline scan (n=3), technical failure (n=1), staff unavailability (n=1) and failure to save the primary imaging for analysis (n=1). For the sixty-four patients included in this analysis, two DCE- MRI scans were acquired a mean of 3.7 days apart, (range 2-7 days). The number of lesions used to calculate the reproducibility of each parameter are given in Table 17, and varied between 111 and 119 paired datasets depending on parameter.

The distributions of median parameter values from each lesion were acquired from scans at the second baseline visit and analysed using histograms. The histograms of median parameter values for: WTV, EF, T1, IAUC-60, K^{trans}, Ve, Vp and ADC are shown in Figure 6 A-G. The distributions of whole tumour volume and enhancing fraction as shown in Figures 6A and 6B were highly skewed and were clearly not normally distributed. The median values of T1 shown in Figure 7C were consistent with a normal distribution as shown by the results from the normality test (p=0.748).

Although the median IAUC values for visit 2 in Figure 6D were not consistent with a Gaussian distribution (p=0.03), the differences between the 2 baseline visits were consistent with a Gaussian distribution (data not shown). None of the parameter distributions for K^{trans}, Ve, Vp or ADC conformed to a Gaussian distribution. The parameters K^{trans} and *Vp* were both highly positively skewed (Figures 6E and 6G). Normality tests confirmed that median values of V_e and ADC were not from a Gaussian distribution (Figures 6F and 6H).

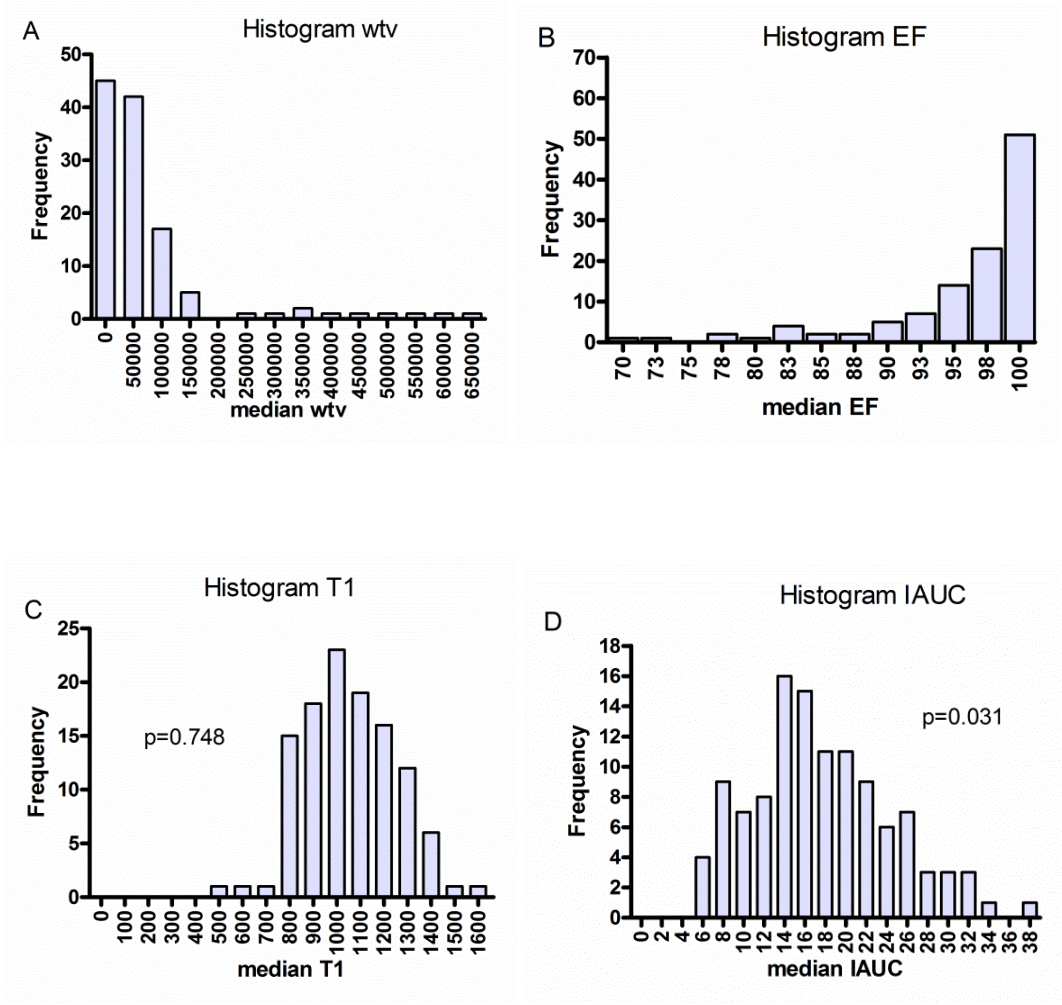

Figure 6: Distribution of median DCE-MRI parameters:

Histograms showing the frequency distribution of DCE-MRI median parameter values: (A) Whole tumour volume (WTV) (B) Enhancing fraction (EF) (C) T1 and (D) IAUC60. Median values were obtained from the second baseline MR scan (prior to therapy). The p values indicate where distributions were tested for normality using the Kolmogorov-Smirnov normality test.

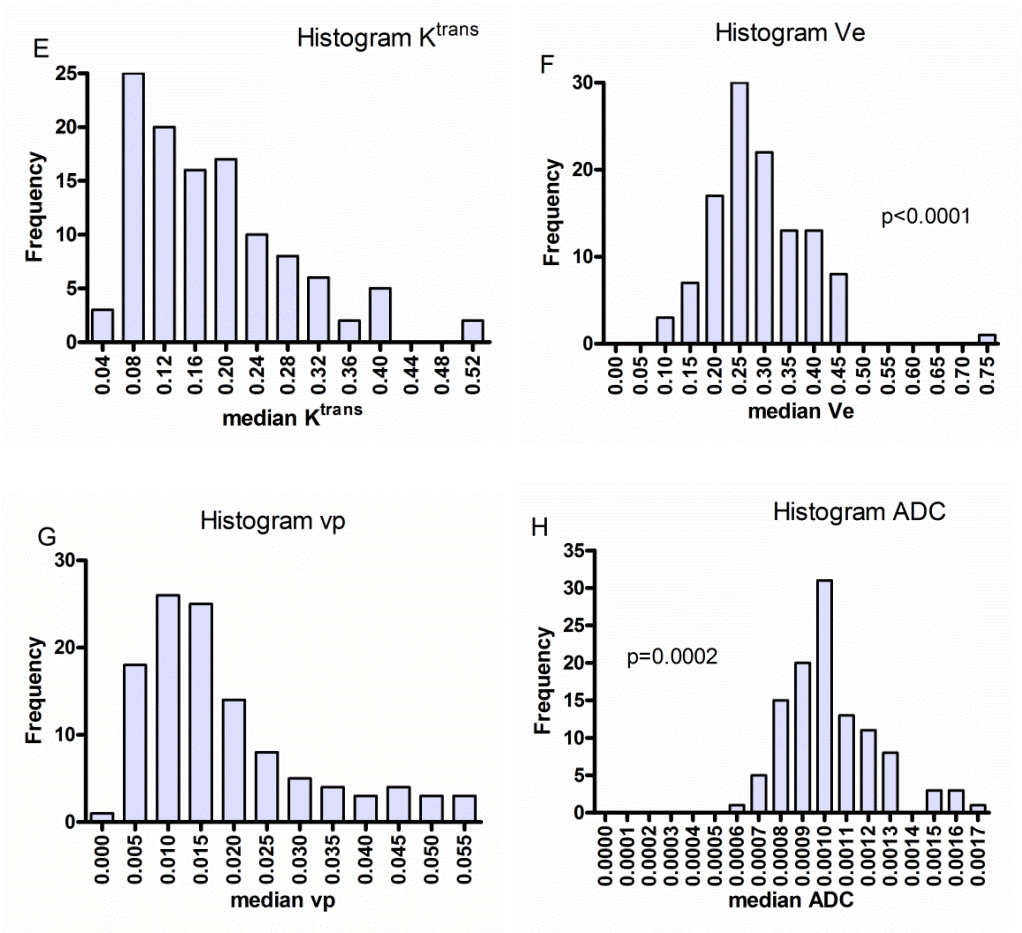

Figure 6 (E-H): Distribution of median DCE-MRI parameters:

Histograms showing the frequency distribution of median parameter values for DCE-MRI and DWI derived parameters: (E) K^{trans}, (F) V_e, (G) *Vp* and (H) ADC. Median values were obtained from the second baseline MR scan (prior to therapy). The p values indicate where distributions were tested for normality using the Kolmogorov-Smirnov normality test.

The Bland-Altman plots for each parameter are shown in Figure 7. These plots were generated using paired data from both pre-treatment scans. The difference between pre-treatment measurements for each variable was plotted against the parameter average.

The plots for WTV and EF show evidence of a dependency between the average parameter value and the parameter difference, indicating a need for transformation of these data. Figure 7A that the parameter difference for WTV was dependant on the average value and this can also be observed in Figure 7B in the Bland-Altman plot for EF. The distribution of the differences for T1 (Figure 7C) and IAUC (Figure 7D) were homogenous, consistent with values obtained from a normal distribution. The presence of value dependency was excluded for parameters T1 and IAUC by the absence of any significant correlation between average values and differences for each parameter.

Parameter value dependency was observed in relation to K^{trans} (Figure 7E) and *Vp* (Figure 7G). For both of these parameters the size of the difference was dependant on the average parameter value. The Bland-Altman plots constructed for Ve (Figure 7F) and ADC (Figure 7H) were also not homogenous.

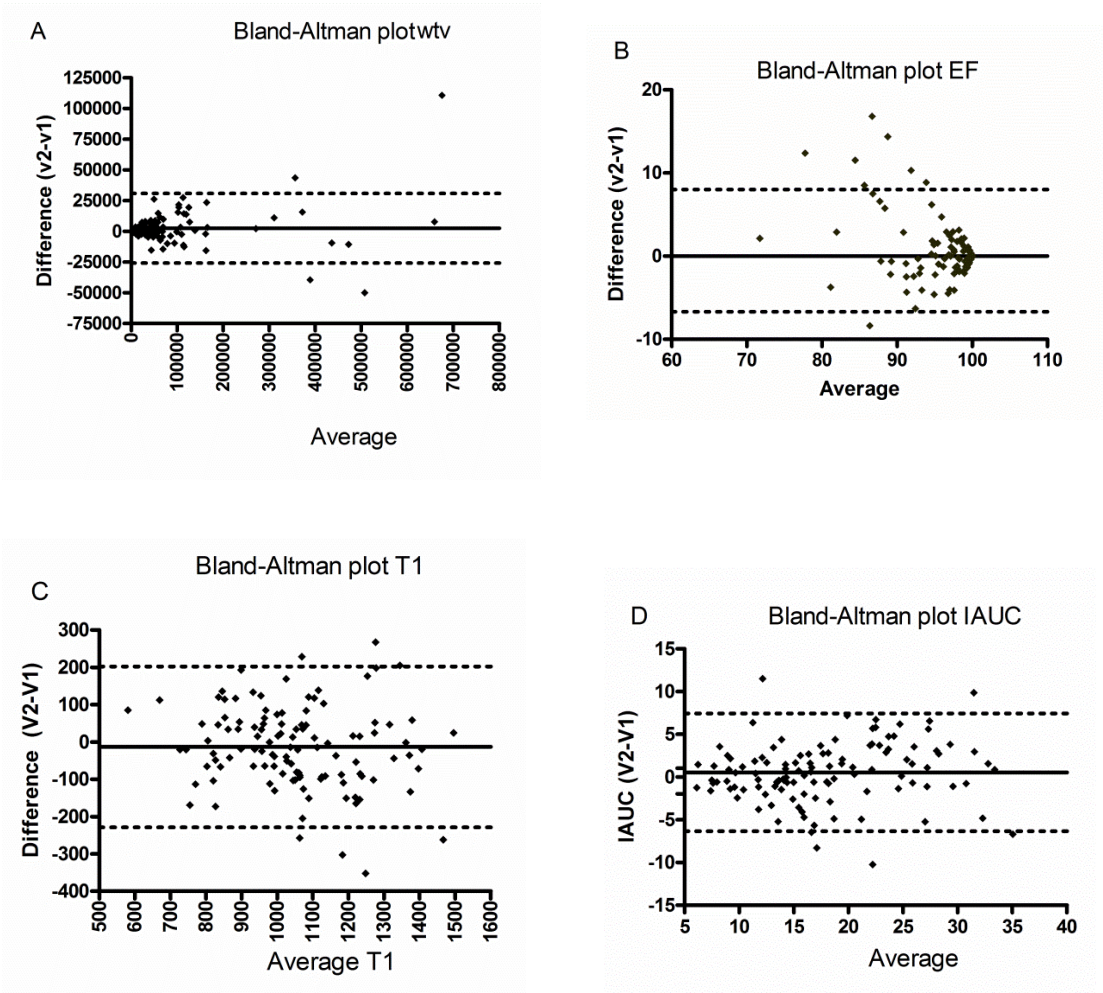

Figure 7: Reproducibility of baseline DCE-MRI parameters.

Bland-Altman plots of paired median values of parameters calculated from pre-treatment scans: (A) WTV; (B) EF; (C) T1 and (D) IAUC.

The average parameter difference between the two visits is indicated by the horizontal solid line. The dotted lines indicate the 95% confidence interval for the mean difference.

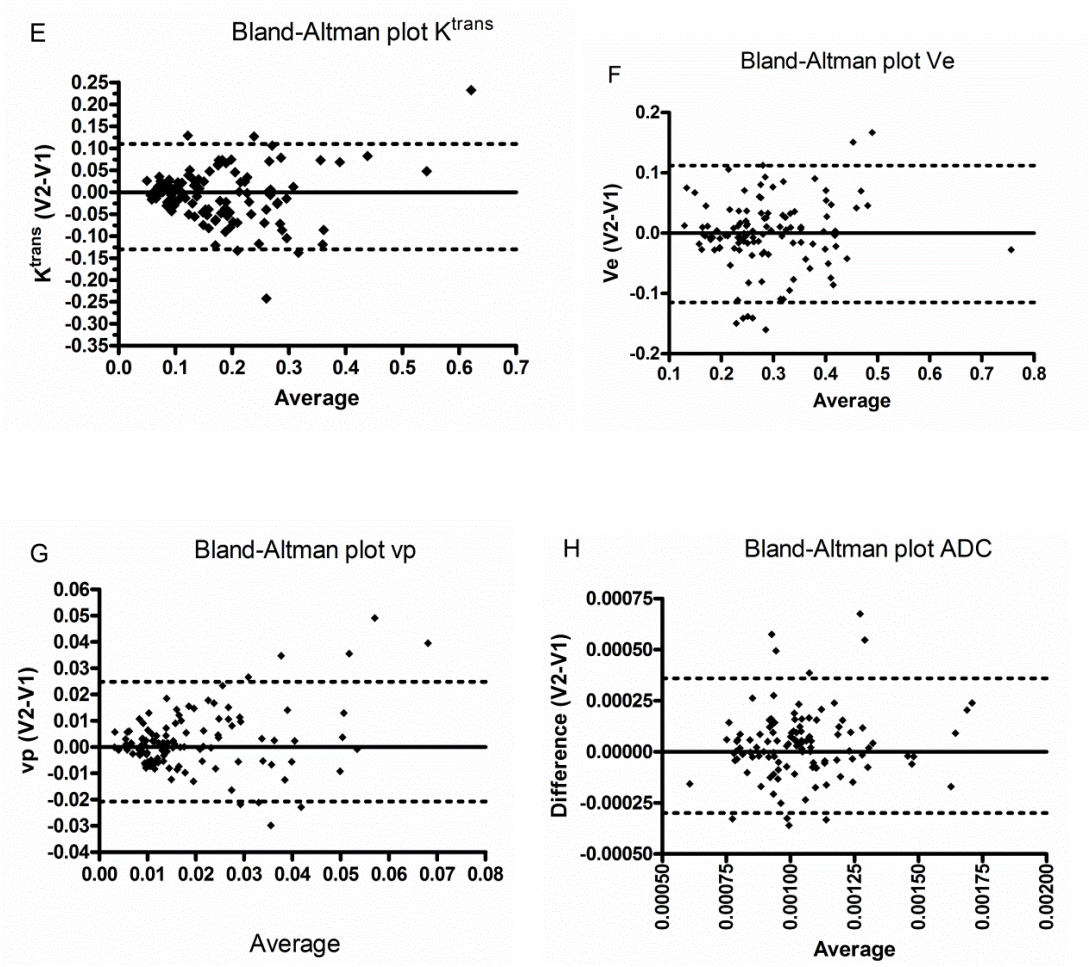

Figure 7 (E-F): Reproducibility of baseline DCE-MRI parameters.

Bland-Altman plots of median parameter values calculated from paired pre-treatment scans: (E) K^{trans}, (F) V_e, (G) *Vp* and (H) ADC.

The average parameter difference between the two visits is indicated by the horizontal solid line. The dotted lines indicate the 95% confidence interval for the mean difference.

These findings indicated that further transformation of the data was necessary for parameters: WTV, EF, K^{trans}, V_e, V_p and ADC. As most of these data were positively skewed, a log normal transformation was initially carried out. Following consultation with a statistician, alternative transformations were necessary for the parameters EF and Vp. The Enhancing Fraction (EF) is expressed as a percentage and data are highly skewed due to the fact that most values are 95-100%. This data required a more complex transformation (log (101-EF)). A square root transformation was applied to Vp. The same transformations were consistently applied throughout the data analysis for each parameter.

Bland-Altman plots of the transformed parameter data for log WTV, log (101-EF), log K^{trans}, log Ve and √Vp are presented in Figure 8. In each case, transformation of the parameter value resulted in a more homogenous distribution. There was no evidence of a correlation between parameter differences and the parameter average, which excluded any value dependency.

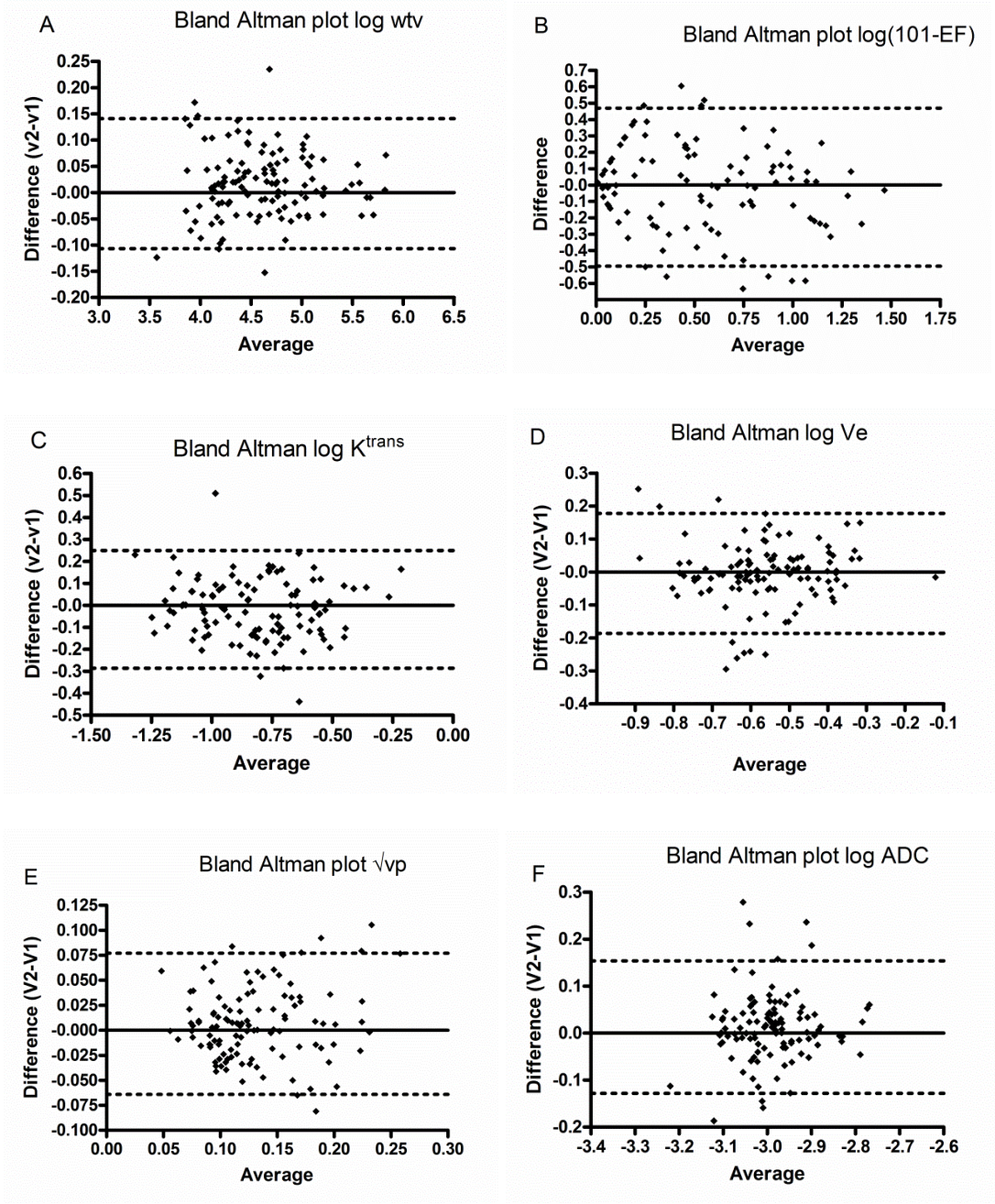

Figure 8: Reproducibility of transformed DCE-MRI parameters

Bland-Altman plots for transformed parameters (A) log WTV; (B) log (101-EF); (C) log K^{trans}; (D) log Ve (E) √Vp and (F) log ADC.

The average parameter difference between the two visits is indicated by the horizontal solid line. The dotted lines indicate the 95% confidence interval for the mean difference.

Reproducibility statistics for DCE-MRI and DWI parameters were calculated using a Bland-Altman method and are presented in Table 17. Most of the cohort of 70 patients had more than one tumour in the MR scan field of view, a total of 119 tumours were included in these analyses. The most common reason for the exclusion of data was excessive patient motion, resulting in inaccurate parameter values.

The co-efficient of reproducibility describes the variation in the difference between paired measurements. It can therefore be viewed as a threshold for determining the significance of change. Whilst changes smaller than this value could be related to normal variation and error: in contrast larger changes can be considered to be due to biological change. The parameter with the poorest co-efficient of reproducibility was Vp where the co-efficient of reproducibility was equal to the mean parameter value, indicating a high variability for this parameter. These values indicate that a significant change in most parameters would be required in order to be confident that changes were related to underlying biology.

The within-patient co-efficient of variation was calculated for each patient using a previously published method used by our group. The parameter with the lowest within-patient CV was EF (2.8%) and V_p had the highest within-patient CV (41.6%).

Parameter	N	Mean (visit 2)	Bias**	95% limit of agreement	***CR	Within patient CV#
WTV(mm^3)	119	82,283	2566	-25,979 to 30,930	28,363	12.6%
EF (%)	113	96.32	0.63	-6.72 to 7.97	7.4	2.8%
IAUC ($mmol.min^{-1}$)	114	18.16	0.54	-6.34 to 7.43	6.9	13.8%
K^{trans} (min^{-1})	114	0.17	-0.01	-0.13 to 0.11	0.12	25.7%
V_e	114	0.29	-0.00	-0.12 to 0.11	0.12	14.0%
V_p	114	0.02	0.00	-0.02 to 0.02	0.02	41.6%
T1(ms)	114	1045	-13.40	-229 to 202	216	7.5%
ADC(mm^2/s)	111	$1x\ 10^{-3}$	$0.034\ x10^{-3}$	$-0.30x10^{-3}$ to $0.36x10^{-3}$	$0.34\ x\ 10^{-3}$	12.1%

Table 17: Reproducibility of DCE-MRI parameters.

N = number of paired tumour assessments; SD = standard deviation

*Parameter mean values were calculated from individual median values at the second baseline visit. **The bias refers to the mean difference in the parameter between the two baseline visits. ***The co-efficient of reproducibility (CR) =1.96 x SD. #CV: coefficient of variation

4.2.2 MR biomarkers identify intra- and inter-patient heterogeneity in patients with colorectal liver metastases

This section of the thesis tests the hypothesis that DCE-MRI and DWI-MRI can detect differences between metastases in different subjects and between metastases in an individual subject. This would help to develop further our understanding of the role for DCE-MRI and DWI in personalised treatment strategies.

In order to control for the effects of the tumour microenvironment, only patients with multiple liver metastases were included in this analysis. Twenty-five subjects bearing 73 tumours were eligible for inclusion.

Figure 9 overleaf shows example parametric maps acquired at two baseline visits for a range of DCE-MRI and DWI-MRI parameters.

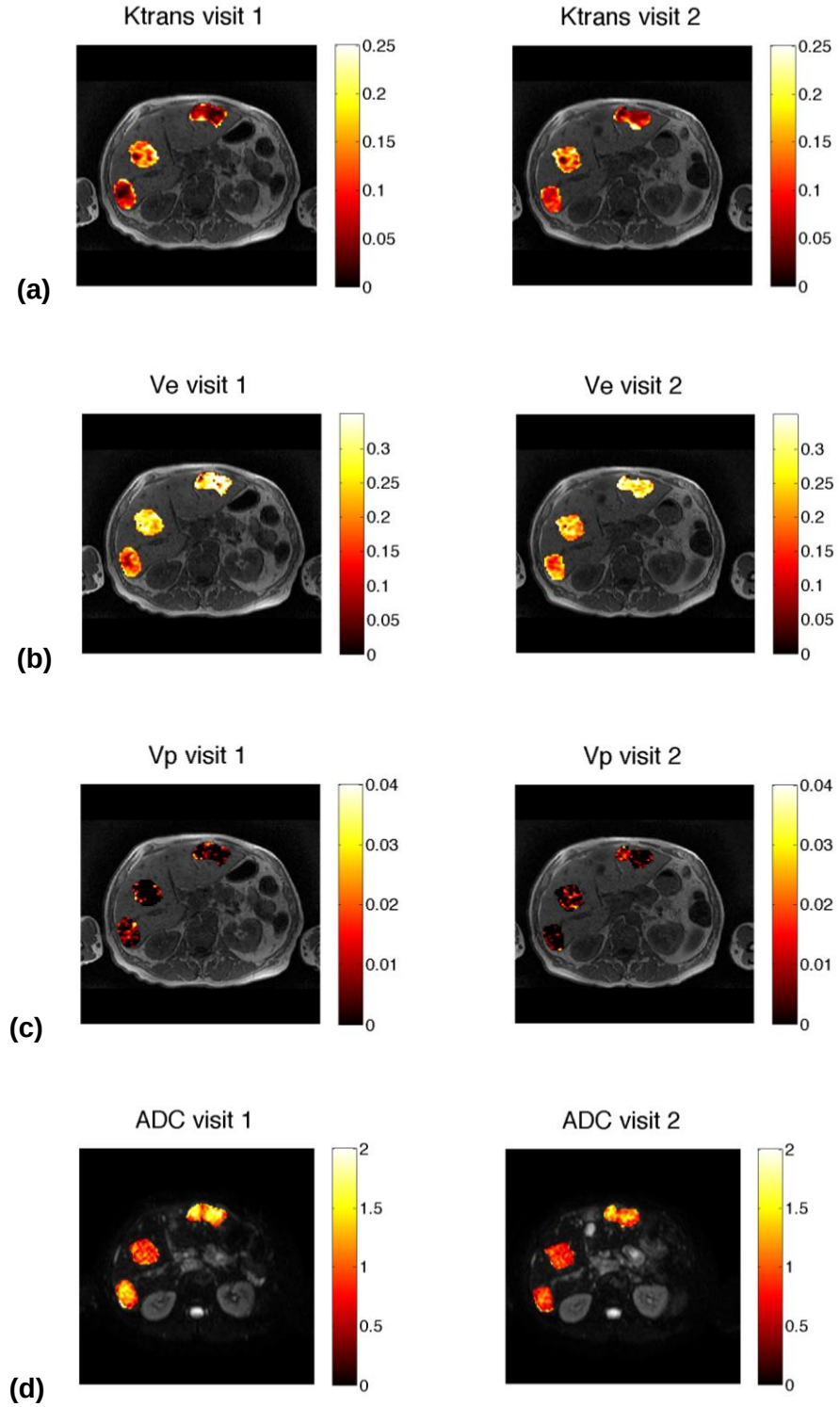

Figure 9: Parametric maps from a patient with multiple liver metastases on 2 separate baseline visits.

(a) K^{trans} (b) V_e (c) V_p and (d) *ADC*. Images are from a 74 year old man with multiple liver metastases secondary to metastatic colorectal cancer.

4.2.2.1 Parameter distributions in patients with liver metastases

Histograms for median values of each parameter (K^{trans}, V_e, V_p, EF and ADC) from the first pre-treatment visit are shown in Figure 10. These histograms demonstrate considerable heterogeneity for each variable. The spread of the variable relates to the associated information content.

In order to establish the reproducibility of parameters, Bland-Altman plots were constructed. These demonstrate high variation between the two pre-treatment scans and show that the difference in parameter values is related to the mean parameter value. Bland-Altman plots are shown in Figure 11.

In order to carry out further statistical analysis it was necessary to transform the variables. These transformations and associated measurement errors and standard deviations are shown in Table 18 together with the non-linear functions used to generate transformed variables with approximately Gaussian distribution. The standard deviations of these distributions are also shown.

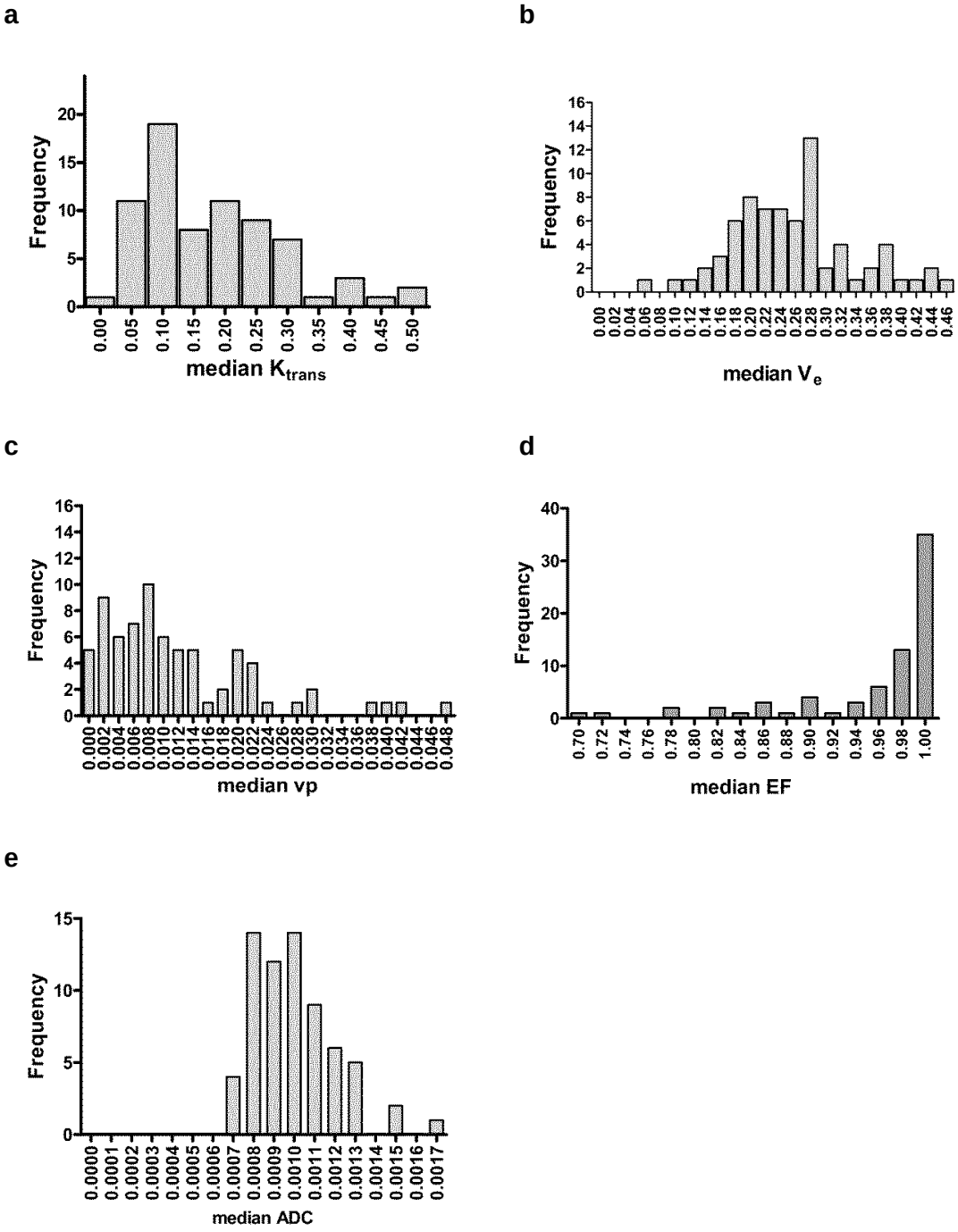

Figure 10: The distribution of median DCE-MRI variables from first baseline visit.

(a) K^{trans} (b) $V_{e;}$ (c)*Vp* (d) EF and (e) ADC.

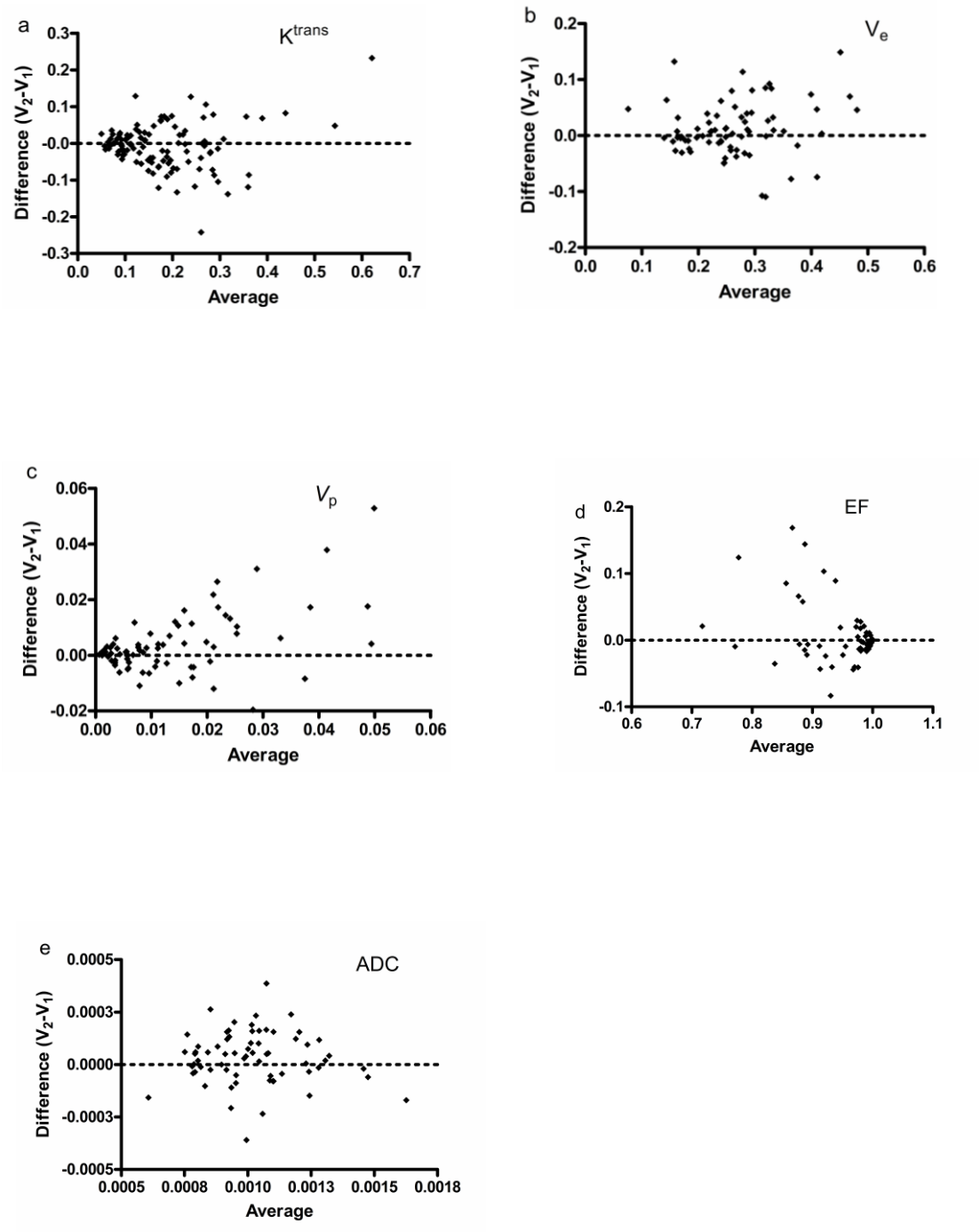

Figure 11 Bland-Altman plots showing reproducibility of median values for each parameter in the subset of patients with multiple liver metastases.

(a) K^{trans} (b) V_e (c) V_p (d) EF and (e) ADC.

Parameter x	Error model	Transformation y	Standard deviation σ_y
K^{trans}	$x \pm k$	$y = -log(x)$	0.29
V_e	$x \pm kx$	$y = -\log(x)$	0.16
V_p	$x \pm kx^{1/2}$	$y = \sqrt{x}$	0.038
EF	$x \pm k\sqrt{(x - x^2)}$	$y = \text{asin}(2x - 1)$	0.17

Table 18: Approximate error models determined from reproducibility data, and the transformations needed to produce approximate Gaussian random variables.

Bland-Altman plots of the transformed parameters scaled to the associated signal content are shown in Figure 12. The Bland-Altman plots of the transformed variables show a more uniform reproducibility, indicating that the transformation had been appropriate. For a successful transformation the residual distributions (distribution of scatter above and below zero) should be independent of the variable. The highly skewed nature of the EF plot is due to the quantization of this variable at 100%, which causes identical values that cannot be separated by a transformation.

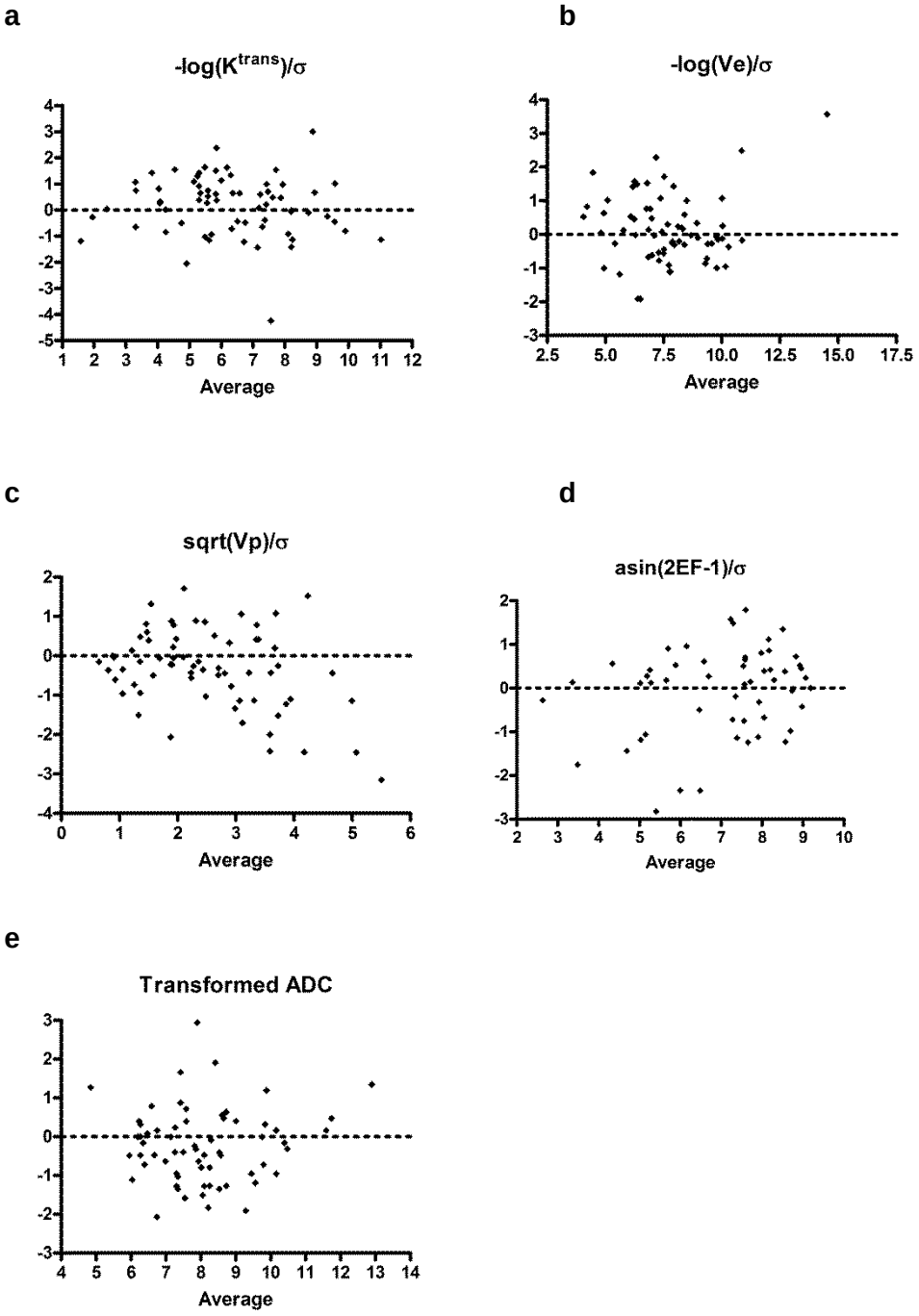

Figure 12: Bland-Altman plots, showing reproducibility of transformed variables.

Displayed variables are (a) K^{trans} (b) V_e (c)V_p (d) EF and (e) ADC (top left to bottom right) scaled on the x axis to units of measured reproducibility.

Gaussian random variables containing no signal (only noise) are expected to have a Gaussian distribution with unit variance. However larger values indicate the amount of biological variation implicit in this measurement as shown in Table 19 and illustrated in Figure 13.

The degree of uniformity of reproducibility in these plots (now within a factor of two) demonstrates the utility of the chosen mapping functions. Four of the 73 repeat baseline data sets were found to have a chi-square of 16 for 5 degrees of freedom, indicating that there had been a problem with obtaining equivalent repeat measurement. As we could not determine which of these was in error, both of the baseline measurements were excluded from further study. The distribution of D for the reproducibility data was then found to have a mean of 1.006, which is very close to the theoretical value of 1.0 for independent y^i.

The statistical distances D for: a) differences between tumours within individual subjects and b) differences between tumours from different subjects gave mean values of 1.39 (+/-0.099, +/-0.06) and 2.23 (+/- 0.027, +/- 0.10), respectively. Both of these values were significantly different to the null hypothesis that the data can be accounted for by measurement error ($p< 10^{-6}$). These results demonstrate that there was statistically significant heterogeneity in liver metastases between subjects which was more marked than that between multiple liver metastases in a given subject. However, the combination of imaging biomarkers was also sufficient to detect a statistically significant difference between individual liver metastases in subjects with multiple lesions.

Parameter	Transformation	Signal SD
K^{trans}	$y = -log(x)$	3.14
V_e	$y = -log(x)$	2.67
V_p	$y = \sqrt{x}$	1.96
EF	$y = asin(2x - 1)$	2.48

Table 19: Signal content of DCE-MRI variables.

Signal content measured as the SD of the corresponding transformed Gaussian random variable (as shown in Figure 13).

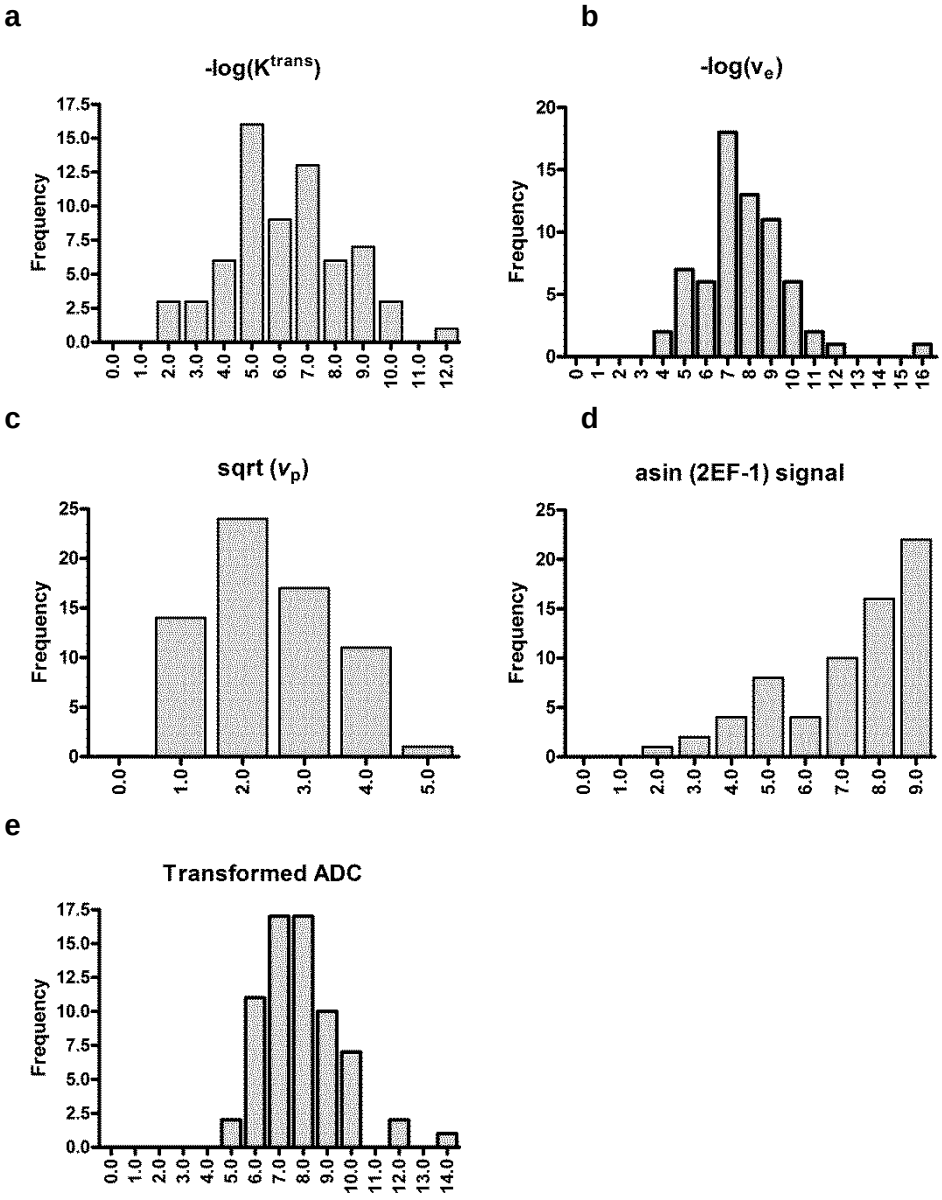

Figure 13: **Transformed DCE-MRI variables scaled to reproducibility.**

Unlike the original parameter distributions, the spread of each variable is a measure of the associated information content.

(a) K^{trans}, (b) V_e, (c)V_p (d) EF and (e) ADC

4.2.3 Imaging parameters and clinical factors

4.2.3.1 Pre-treatment whole tumour volume correlates with serum LDH

Correlations were explored between WTV and selected clinical prognostic factors, these results are shown in Table 20. There was a highly significant relationship between serum LDH and WTV (Kendall's test, $p<0.0001$). There was no evidence of a significant correlation between tumour volume and platelet count (Pearson's test, $p=0.09$) or between tumour volume and CEA (Kendall's test, $p=0.94$). A scatter plot of serum LDH and WTV is shown in Figure 14.

Clinical prognostic factor	Correlation coefficient (p-value)
platelet count	r=0.2 (p=0.090)
LDH	r=0.47 (p<0.0001)
CEA	r=-0.009 (p=0.944)

Table 20: Correlation between WTV and prognostic indicators

Data represent Pearson correlation coefficients (r) for log WTV and selected prognostic factors in metastatic colorectal cancer. Log normal values of LDH and platelet count were used. All p-values were 2-sided.

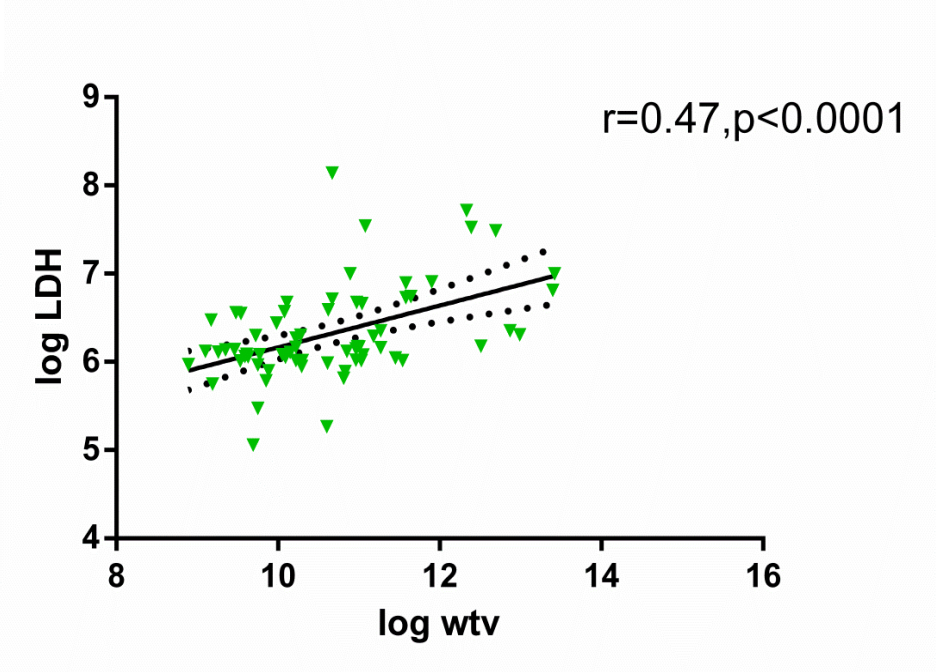

Figure 14: relationship between WTV and serum LDH.

Scatter plot of log whole tumour volume (WTV) and log serum LDH. The solid bar indicates the regression co-efficient with 95% confidence intervals represented by dotted lines. Pearson correlation coefficient (r) and significance level (p) are also shown.

4.2.4 Evidence of correlation between DCE-MRI imaging parameters

4.2.4.1 IAUC and K^{trans}

There was evidence of highly significant association between log K^{trans} and IAUC, r=0.83, p<0.0001 (Pearson's test), shown in Figure 15.

4.2.4.2 K^{trans} and WTV

There was evidence of a negative relationship between K^{trans} and WTV, with increasing tumour volume associated with reduced levels of K^{trans}, r=-0.370 (p=0.0019), Pearson's test, as demonstrated in Figure 16. The relationship between IAUC and WTV was also significant, r=-0.192 (p=0.02), Kendall's test.

4.2.4.3 V_e and ADC

Given that parameters V_e and ADC should both relate to the extracellular extravascular space (EES), it was hypothesized that these two biomarkers might be correlated. However, there was no evidence of a significant relationship between V_e and ADC, r=0.188, (p=0.05, Pearson's test).

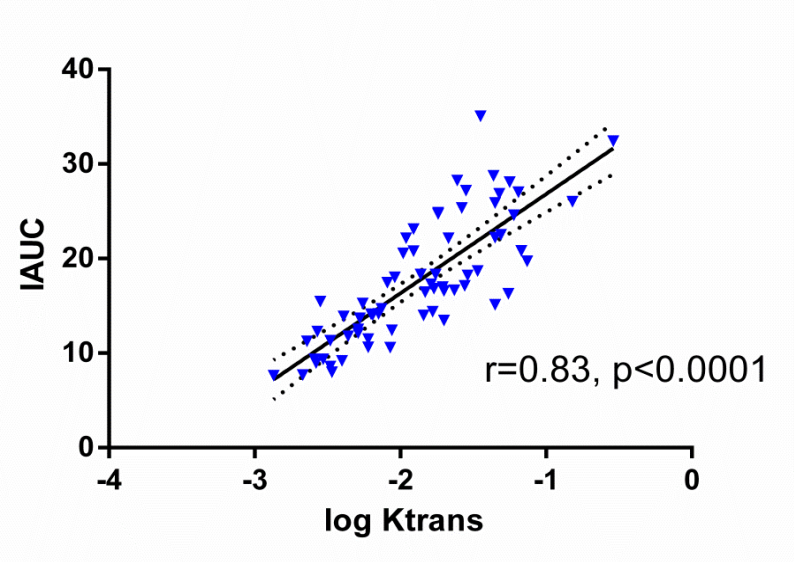

Figure 15: Correlation between log K^{trans} and IAUC.

Scatter plot of log K^{trans} against IAUC. The solid bar indicates the regression co-efficient with 95% confidence intervals represented by dotted lines. Pearson correlation coefficient (r) and significance level (p) are also shown.

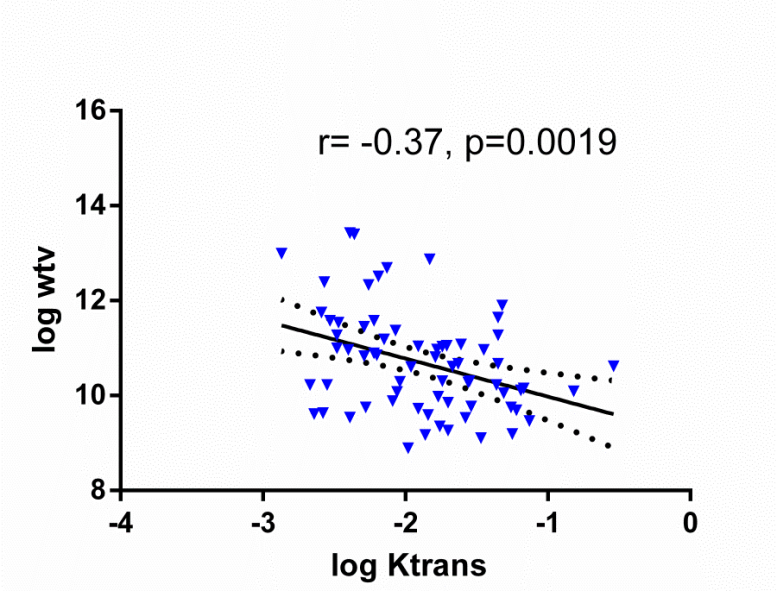

Figure 16: Correlation between log Ktrans and WTV.

Scatter plot of log K^{trans} against IAUC. Log normal transformations of each parameter were used for statistical analysis. The solid bar indicates the regression co-efficient with 95% confidence intervals represented by dotted lines. Pearson correlation coefficient (r) and significance level (p) are also shown.

4.2.5 Correlation of baseline imaging parameters with outcome

4.2.5.1 Baseline DCE-MRI parameters and progression free survival

A univariate Cox regression method was used to investigate the relationship between baseline measurements of each DCE-MRI parameter (as a continuous variable) against the trial end point, progression free survival. There was no significant relationship between DCE-MRI parameters measured at baseline and progression free survival. This is shown in Table 21.

This analysis was repeated for DCE-MRI parameters dichotomised by median value, and these data are presented in Table 22. As with the continuous data, there was no significant difference in progression free survival according to DCE-MRI parameters dichotomised by median value.

DCE-MRI parameter	HR (95% CI)	p-value
*K^{trans}	0.7 (0.4-1.1)	p=0.13
*V_e	0.5 (0.2-1.2)	p=0.10
*V_p	0.0 (0.0-3.0)	p=0.11
*WTV	1.2 (1.0-1.6)	p=0.09
*ADC	1.1 (0.2-5.7)	p=0.92
EF	1.0 (0.9-1.0)	p=0.3
IAUC-60	1.0 (0.9-1.0)	p=0.06
T1	1.0	p=0.24

Table 21: Baseline DCE-MRI parameters and association with progression-free survival.

*****Indicates where transformed values of variables were used for the analysis. Hazard ratio (HR) <1.0 favours low values of the biomarker.

DCE-MRI parameter	median value	Median PFS (wks) (low v high)	HR (95% CI)	p-value
*K^{trans} (min^{-1})	0.159	35 v 48 weeks	1.0 (1.0-2.7)	p=0.06
*V_e	0.289	39 v 43 weeks	1.1 (0.7-1.9)	p=0.55
*V_p	0.014	41 v 40 weeks	1.3 (0.8-2.2)	p=0.28
*WTV (mm^3)	40,946	43 v 37 weeks	0.7 (0.5-1.2)	p=0.23
*ADC (mm^2/s)	0.001	41 v 38 weeks	1.0 (0.6-1.6)	p=0.98
EF (%)	97.5	38 v 46 weeks	1.3 (0.8-2.1)	p=0.31
IAUC-60 ($mmol.min^{-1}$)	16.6	37 v 48 weeks	1.5 (0.9-2.5)	p=0.13
T1 (ms)	1058	46 v 38 weeks	0.6 (0.4-1.0)	p=0.07

Table 22: Dichotomised DCE-MRI parameters and association with progression-free survival.

Baseline DCE-MRI parameters were dichotomised according to median values. A univariate cox regression method was used to investigate a relationship with PFS. Transformed values of these variables were used for this analysis, as necessary. Hazard ratio (HR) <1.0 favours low values of the biomarker.

4.2.5.2 Baseline DCE-MRI parameters and radiological response

Radiological response data were available for 68 trial participants at the time of analysis: 31 patients attained a complete or partial response, 29 patients had stable disease and eight patients had disease that had progressed. There was a highly significant association between pre-treatment IAUC-60 and best RECIST response (Kruskal-Wallis test (P=0.0006)). This is shown in Figure 17. The most significant difference in median values of IAUC was observed when comparing patients who had a complete or partial response with patients whose best response was stable disease. Patients who had a complete or partial response had a median IAUC-60 of 19.7mmol.min^{-1}, compared with 13.9 or 12.5 mmol.min^{-1} for stable disease and progressive disease respectively. A difference in median parameter values across RECIST response categories, was also observed for parameters T1 (Figure 18), V_p (Figure 19) and V_e (Figure 20). Other DCE-MRI parameters and the association with best RECIST response are summarised in Table 23.

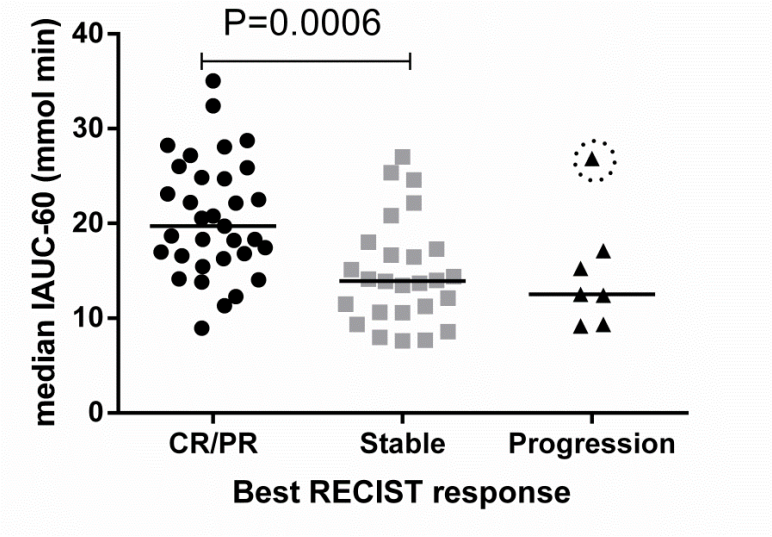

Figure 17: Difference in baseline IAUC-60 according to best RECIST response.

Each point denotes a separate subject. The subject highlighted in the disease progression group represents an outlier. This subject was a 70 year old female whose performance status deteriorated due to treatment related toxicity following the first cycle of chemotherapy and who was subsequently withdrawn from the study. Horizontal bars indicate median values.

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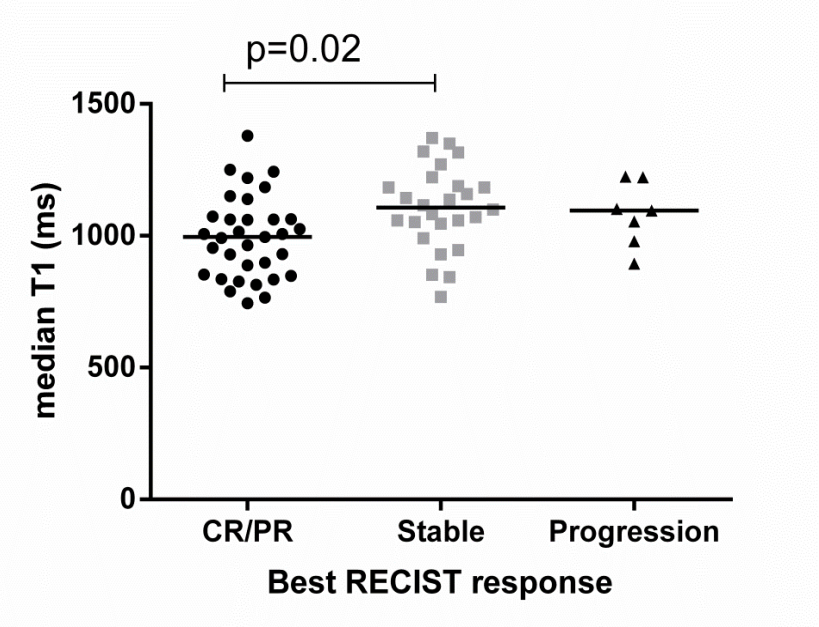

Figure 18: Difference in baseline T1 according to best RECIST response.

Each point denotes a separate subject. Horizontal bars indicate median values.

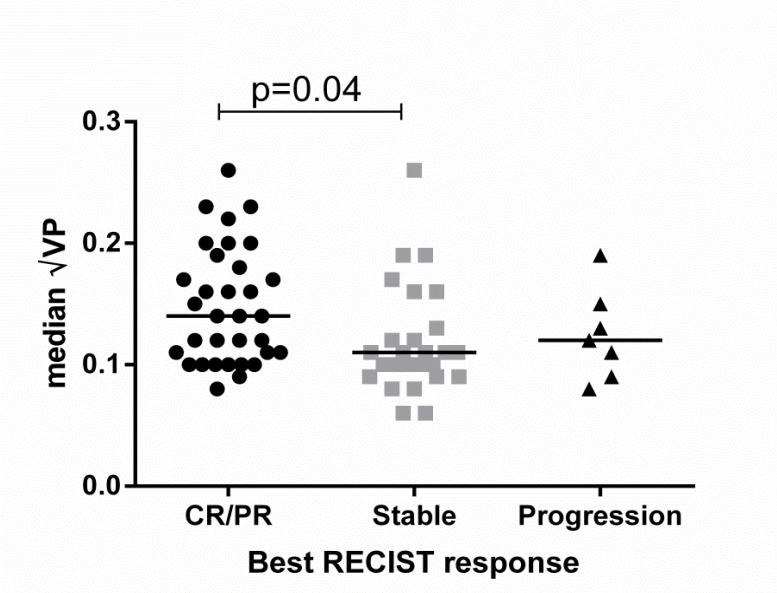

Figure 19: Difference in baseline V_p according to best RECIST response.

Each point denotes a separate subject. Horizontal bars indicate median values.

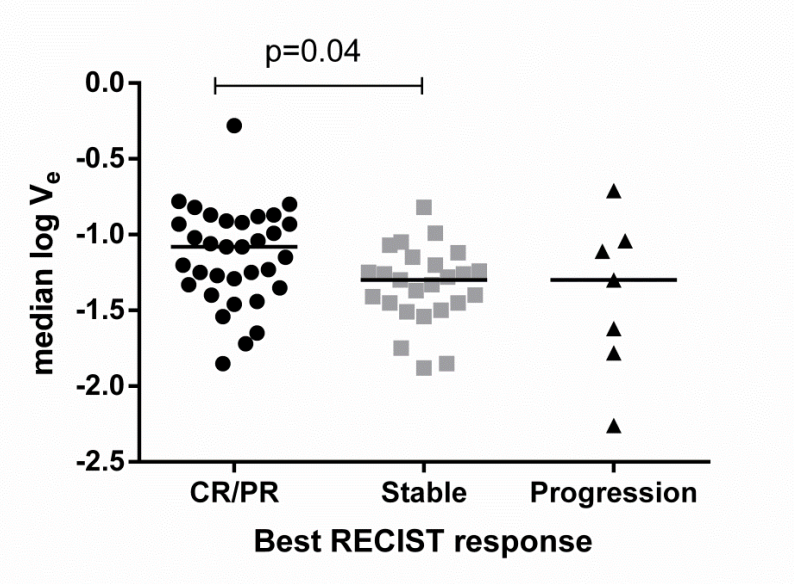

Figure 20: Difference in baseline V_e according to best RECIST response.

Each point denotes a separate subject. Horizontal bars indicate median values.

Baseline variable	Best RECIST response			Two sided p-value
	CR/PR (n=31)	SD (n=29)	PD (n=8)	
	Median values			
WTV(mm^3)**	40135	49020	73130	p=0.12
IAUC60 ($mmol.min^{-1}$)	19.7	13.9	12.5	p=0.0006*
K^{trans} (min^{-1})**	0.181	0.129	0.104	p=0.06
V_e**	0.340	0.273	0.257	p=0.04*
V_p**	0.020	0.012	0.012	p=0.03*
EF**	98.4	97.1	94.2	p=0.18
T1(ms)	996	1107	1096	p=0.02*
ADC**	0.001	0.0012	0.001	p=0.36

Table 23: Median values of parameters according to best RECIST response.

*Indicates a statistically significant result. **Back transformation of parameters was performed to obtain values on a standard ratio scale.

From Table 23 it seems that patients with a high baseline IAUC-60 or K^{trans} prior to treatment were more likely to achieve a complete or partial response, although the difference for K^{trans} was not statistically significant. For both IAUC-60 and K^{trans} there was a step wise reduction in the parameter value moving from complete or partial response to progressive disease. A complete or partial response also appeared to be associated with a larger plasma volume (V_p) and a larger extracellular extra-vascular space (V_e). However there was no clear change in the value of V_p across the response categories, suggesting a more tenuous relationship. Tumour volume was lower in responders although this difference was not significant.

In view of the association between high IAUC-60 and improved RECIST response, IAUC-60 was dichotomised around the median value and progression free survival was compared between the two groups. The median progression free survival for patients categorised according to median value of IAUC-60 was 48 weeks v 37 weeks (Log Rank test, p=0.13). Higher values of IAUC-60 favoured a longer PFS, as shown in Figure 21.

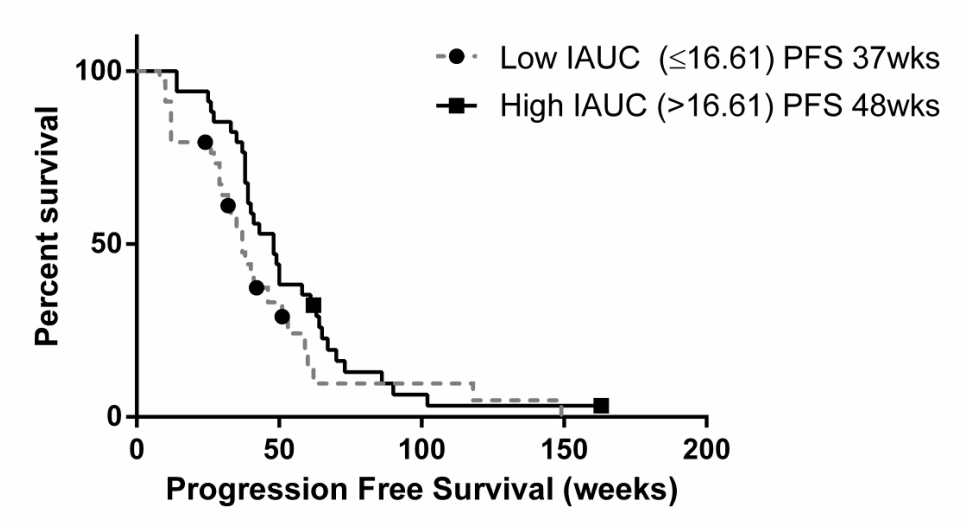

Figure 21: Survival according to IAUC.

Kaplan-Meier estimates of progression free survival according to dichotomisation of IAUC-60 by median values. PFS for subjects with IAUC below the median value (16.61) is shown by the dotted line. PFS for subjects with high IAUC, above 16.61, is shown by the solid line. Censored data are represented by the solid symbols. The difference in the curves was not significant (p=0.13).

4.3 Baseline angiocytokines results

Only a subset of the angiocytokines analysed were included in this thesis. These were selected prior to any analyses involving outcome data.

4.3.1 Reproducibility of angiocytokines

Figures 22 (A-G) illustrate Bland-Altman plots for each of the angiocytokines. In all cases there was evidence of a skewed distribution which was not consistent with a normal distribution. In addition to this, the angiocytokines showed a dependent relationship between the parameter mean and difference with greater differences at higher mean values. In view of this it was necessary to transform the data for further statistical analysis and log normal transformation was carried out on the variable values.

Corresponding Bland-Altman plots of transformed variables are shown in Figure 16 (A-G). In all cases transformation resulted in a more homogenous distribution, indicating the appropriateness of the transformation. It can be observed that some of the variables such as HGF and VEGFR-2 showed a much narrower distribution of values, suggesting less inter-subject variability.

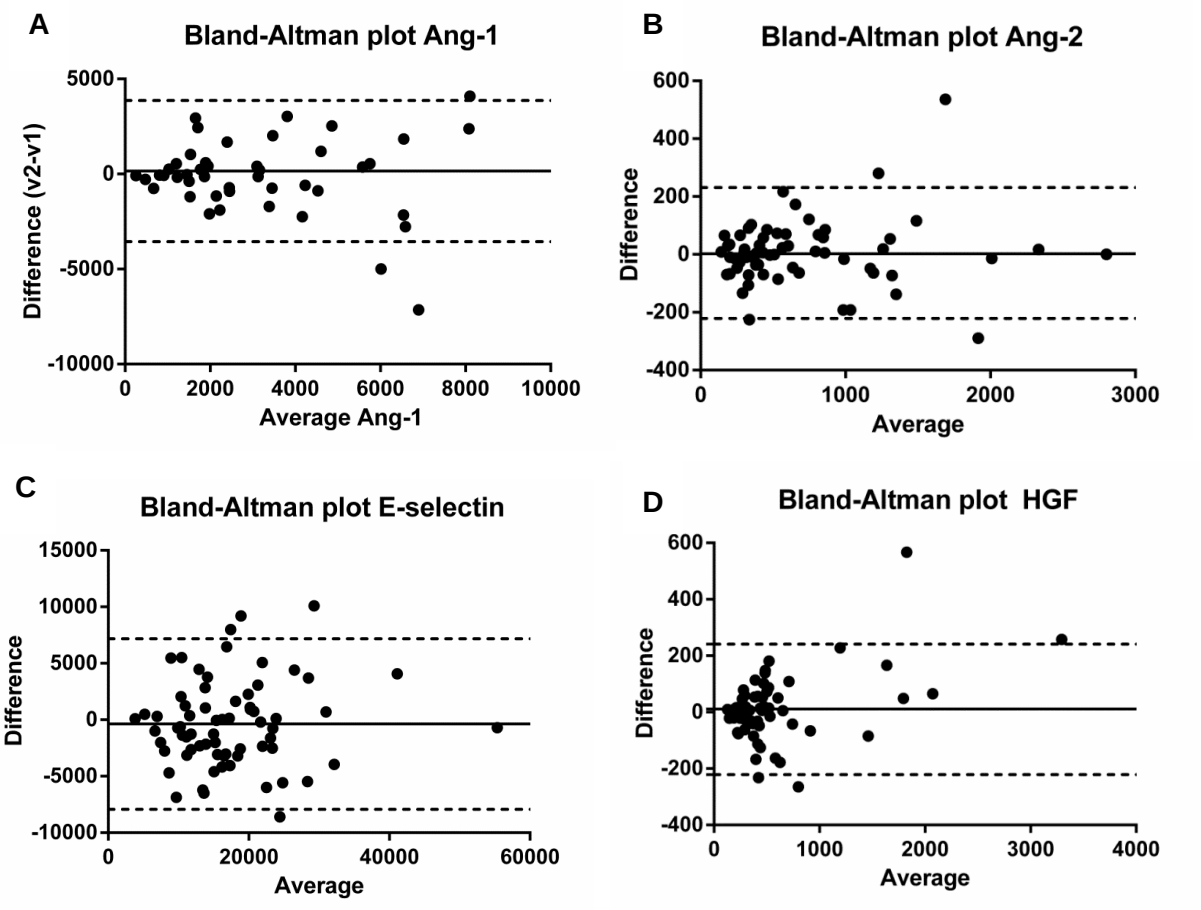

Figure 22: Bland-Altman plots showing the reproducibility of angiocytokines.

Bland-Altman plots depicting: (A) Ang-1 (B) Ang-2 (C) E-selectin (D) HGF.

All units are measured in pg/ml. For each graph, the solid line represents the mean difference between the two visits and the dotted line the 95% confidence interval for the difference.

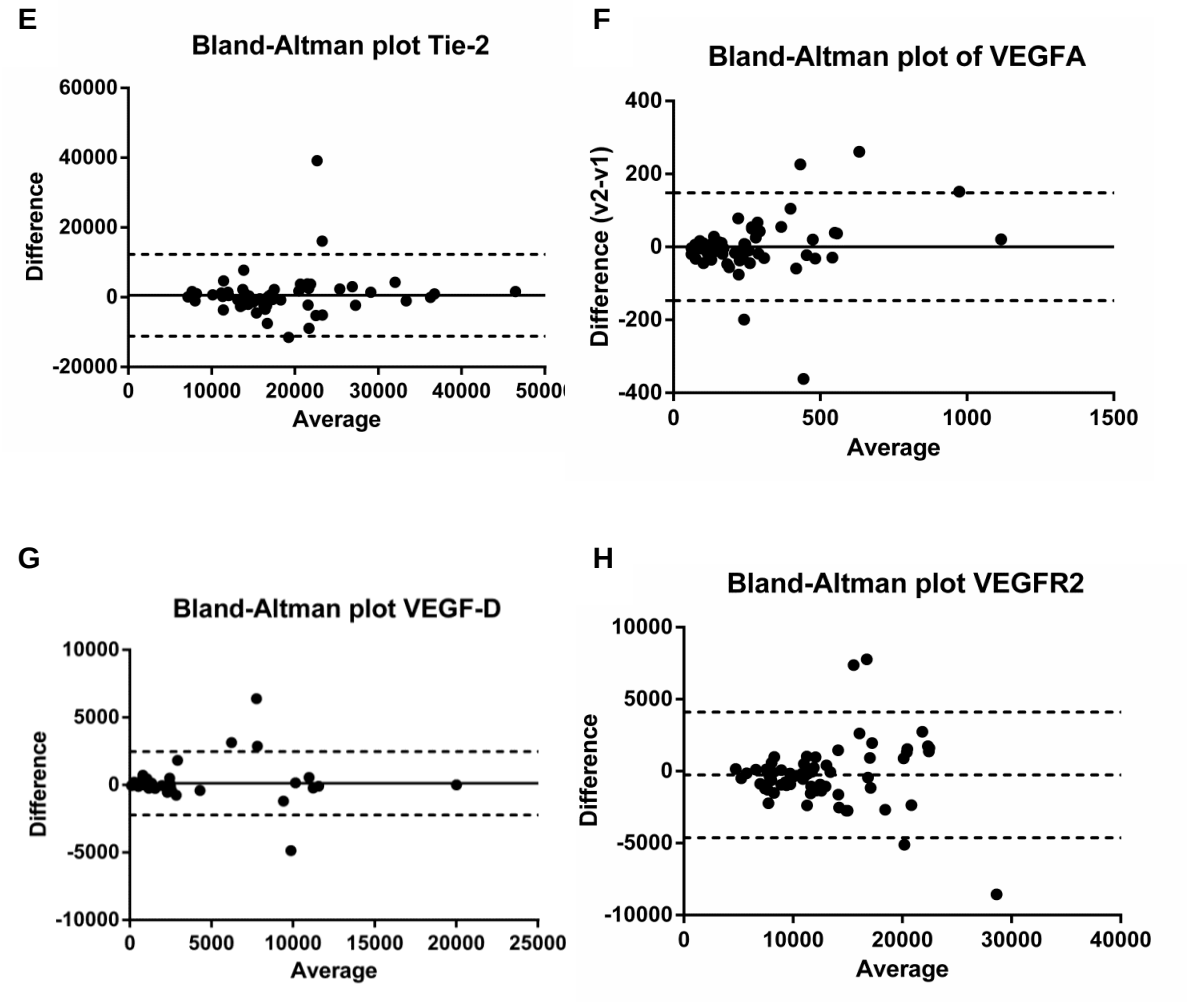

Figure 22 (continued): Bland-Altman plots showing the reproducibility of angiocytokines.

Bland-Altman plots depicting: (E) Tie-2 (F) VGEF-A(G) VEGF-D (H) VEGFR-2.

All units are measured in pg/ml. For each graph, the solid line represents the mean difference between the two visits and the dotted line the 95% confidence interval for the difference.

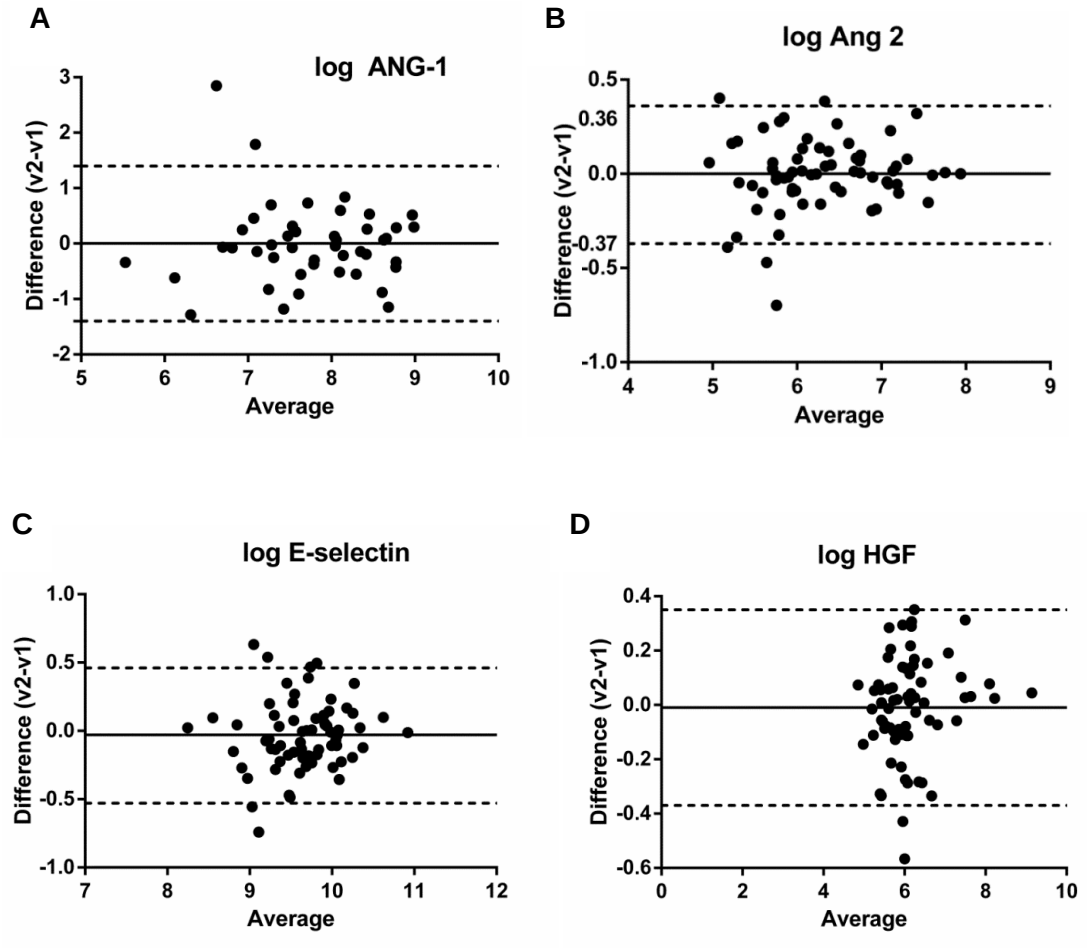

Figure 23: Bland-Altman plots showing reproducibility for log transformed values:

(A) Ang-1 (B) Ang-2 (C) E-selectin and (D) HGF

For each graph, the solid line represents the mean difference between the two visits and the dotted line the 95% confidence interval for the difference.

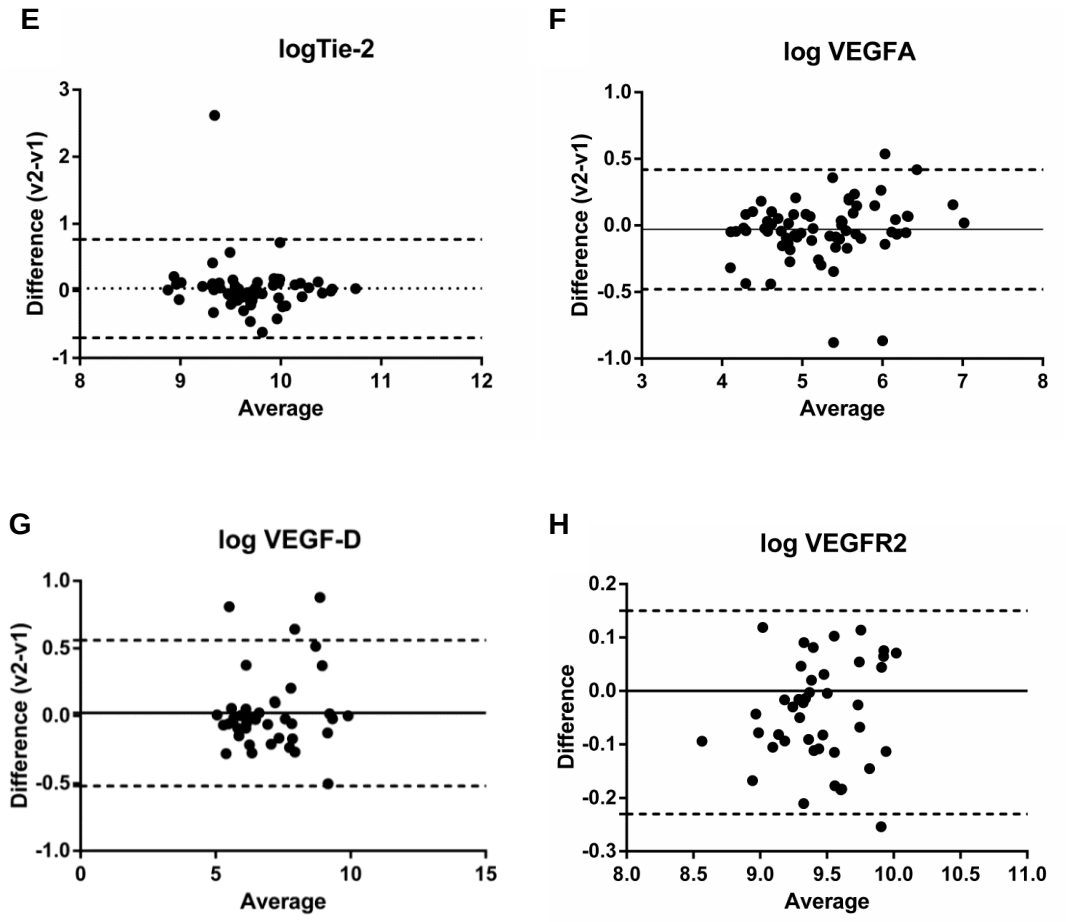

Figure 23 (continued): Bland-Altman plots showing reproducibility for log transformed values:

(E) Tie-2 (F) VEGF-A (G) VEGF-D and (H) VEGFR-2.

For each graph, the solid line represents the mean difference between the two visits and the dotted line the 95% confidence interval for the difference.

4.3.2 Analysis of circulating angiocytokines and patient characteristics

In general, was no evidence of relationship between pre-treatment levels of angiocytokines and age, gender or performance status. However a weak negative correlation was observed for between levels of VEGF-D and performance status. This suggested that higher levels of VEGF-D were associated with a better (lower) performance status. Correlations are shown below in Table 24.

Angiocytokine	**Age** r (p-value)	**Gender**	**Performance status (0-2)**
Ang-1	-0.01 (0.90)	0.17 (0.86)	0.07 (0.45)
Ang-2	-0.11 (0.17)	0.10 (0.30)	0.00 (0.99)
E-selectin	-0.11 (0.20)	-0.13 (0.21)	0.00 (1.0)
HGF	-0.12 (0.14)	0.11 (0.29)	0.19 (0.05)
Tie-2	-0.02 (0.78)	-0.04 (0.67)	0.09 (0.35)
VEGF-A	-0.09 (0.30)	0.11 (0.29)	0.11 (0.24)
VEGF-D	-0.12 (0.14)	-0.01 (0.95)	**-0.23 (0.02)**
VEGFR-2	-0.14 (0.10)	0.02 (0.87)	-0.08 (0.42)

Table 24: Correlations between angiocytokines and clinical factors

4.3.3 Association between baseline angiocytokines and outcome

4.3.3.1 Baseline angiocytokines and PFS

The association between baseline levels of angiocytokines and progression free survival was explored for treatment with bevacizumab and chemotherapy using a univariate cox regression analysis. These results are summarised in Table 25. The relationship between pre-treatment levels of VEGF-A as a continuous variable and progression free survival was significant HR 1.6 (p=0.02). This indicates a significant correlation between absolute levels of VEGF-A and progression free survival, with increasing levels of VEGF-A associated with a poorer outcome. The absence of a significant relationship for the other angiocytokines with progression free survival, for this particular analysis with angiocytokines as continuous variables, is indicated by the Hazard ratios and p values.

In order to further explore the relationship between angiocytokines and progression free survival, pre-treatment concentrations of angiocytokines were dichotomised according to median levels. These results are shown in Table 26. High baseline values of HGF were associated with a worse progression free survival outcome (38 v 51 weeks; p=0.005) compared with patients with a low HGF. Similarly, patients with a high pre-treatment VEGFR-2 had a worse outcome compared to those in the low group (37 v 46 weeks; p=0.02). Surprisingly, the outcome for patients with low versus high pre-treatment VEGF-A was less significant compared with our earlier analysis examining VEGF-A as a continuous variable. Kaplan-Meier plots illustrating the relationship between pre-treatment levels of VEGF-A and HGF and progression free survival, are shown in Figure 24.

Angiocytokine	HR(95% CI)	p-value
Ang-1	1.2 (0.8-1.8)	p=0.26
Ang-2	1.3 (0.9-1.7)	p=0.14
HGF	1.2 (0.9-1.7)	p=0.19
VEGF-A	1.6 (1.1-2.4)	**p=0.02**
VEGF-D	1.1 (1.0-1.2)	p=0.20
VEGFR-2	1.4 (0.8-2.3)	p=0.18
Tie-2	1.4 (0.7-3.1)	p=0.37
E-selectin	1.0 (0.6-1.6)	p=0.86

Table 25: Association between baseline angiocytokines and survival.

Results of univariate cox regression analysis for baseline values of circulating angiocytokines as continuous variables and progression free survival, HR>1.0 indicates a shorter PFS for high vs low values of the biomarker.

Angiocytokine	Median (pg/mL)	Median PFS (wks) (low v high)	HR(95% CI)	p-value
Ang-1	2540	46 v 38	0.7 (0.4-1.2)	0.16
Ang-2	350	40 v 39	0.6 (0.4-1.1)	0.87
HGF	415	51 v 38	0.5 (0.3-0.8)	**0.01**
VEGF-A	190	46 v 37	0.7 (0.4-1.1)	0.1
VEGF-D	651	41 v 40	0.6 (0.4-1.1)	0.1
VEGFR-2	11731	46 v 37	0.5 (0.3-0.9)	**0.02**
Tie-2	15835	40 v 46	1.1 (0.7-1.9)	0.64
E-selectin	16481	39 v 43	1.1 (0.7-1.8)	0.83

Table 26: Baseline angiocytokines and PFS split by median.

Results of univariate cox regression analysis for baseline values of circulating angiocytokines dichotomised according to median value and progression free survival (PFS), Hazard ratio (HR) <1.0 favours low group.

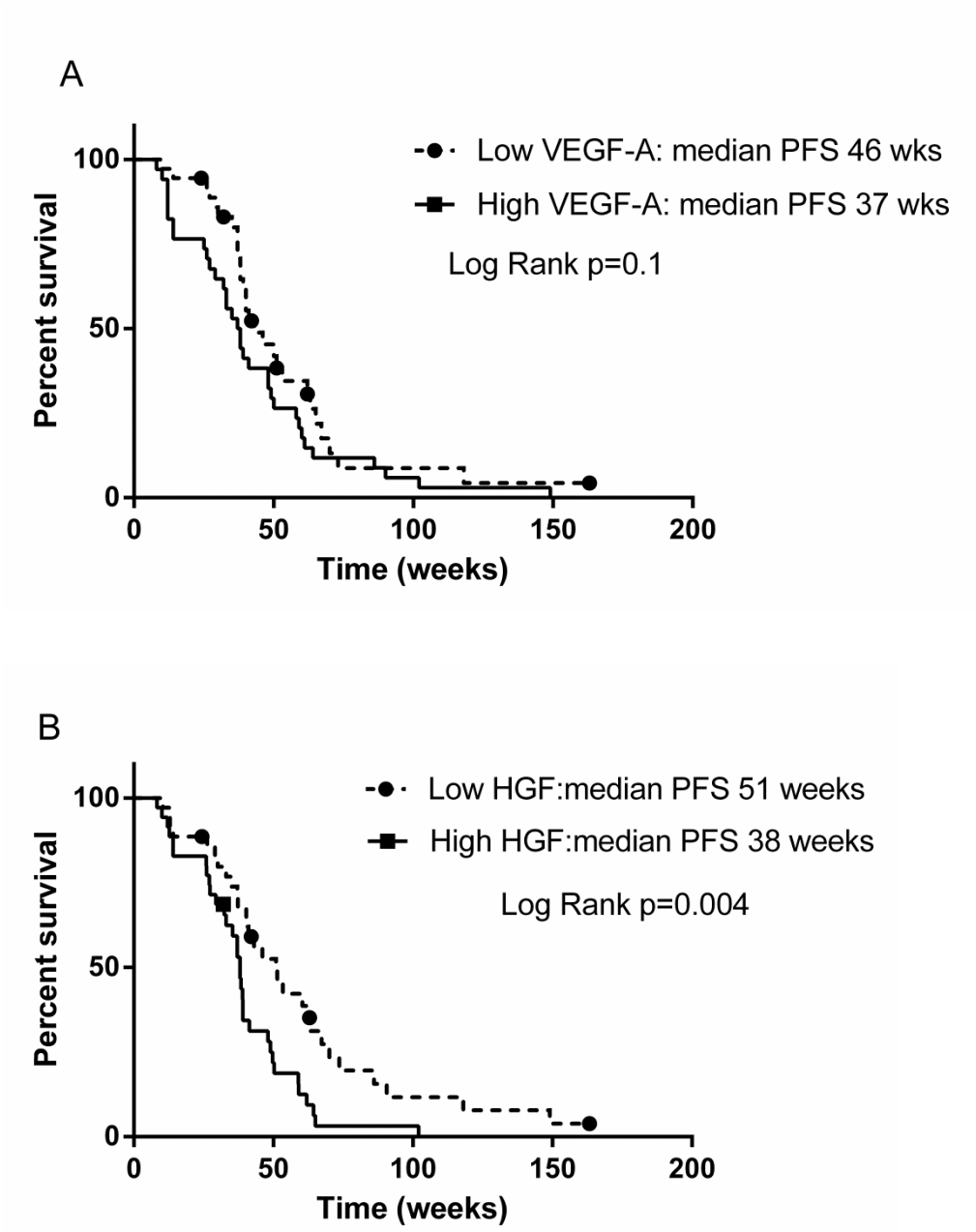

Figure 24: Survival plots based on baseline VEGF-A and HGF.

Kaplan-Meier survival plots of:

(A) Progression free survival for patients with VEGF-A levels above and below the median value of 190pg/mL.

(B) Progression free survival for patients with HGF levels above and below the median value of 415pg/mL.

4.3.3.2 Pre-treatment angiocytokines and radiological response

Pre-treatment levels of angiocytokines were divided into three categories based on the best RECIST response during the trial, which was available for 68 patients. There were 31 patients who had a complete or partial response, 29 patients with stable disease and eight who had progressive disease. Patients whose best response after treatment with bevacizumab and chemotherapy was progressive disease, had higher median levels of Tie-2 at baseline than patients who had stable disease (p=0.027, Kruskal Wallis test). This is illustrated in Figure 25. The differences between median values of VEGF-A, VEGF-D, VEGFR-2, Ang-1, Ang-2, E-selectin or HGF, according to best radiological response were not significant.

Given the association between radiological response and Tie-2, we also explored the relationship between pre-treatment measurements of WTV and Tie-2. Pre-treatment WTV and Tie-2 were significantly correlated (R^2 0.14, p=0.0003) as illustrated in Figure 26. However, there was no correlation between pre-treatment WTV and VEGF-A, VEGF-D, VEGFR-2, Ang-1, Ang-2, E-selectin or HGF.

We hypothesized that there might be a relationship between pre-treatment assessments of DCE-MRI parameters; IAUC, *Vp* and K^{trans} and levels of circulating angiocytokines. We observed a negative correlation between *Vp* and Tie-2 (p=0.02), VEGFR-2 (p=0.04) and VEGF-A (p=0.05), although the latter just reached reach statistical significance. However, there was no evidence of an association between IAUC or K^{trans} and circulating angiocytokines. Scatter plots illustrating the relationship between *Vp*, Tie-2 and VEGFR-2 are shown in Figure 27.

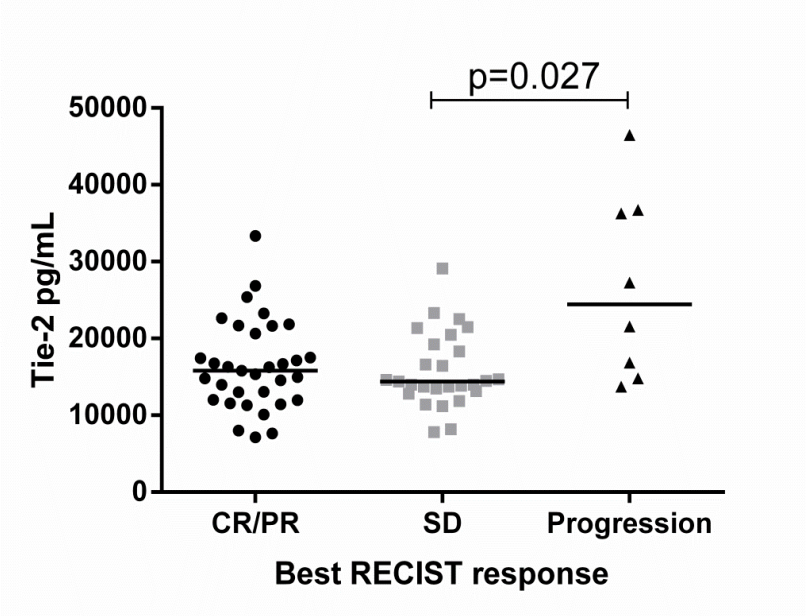

Figure 25: Baseline Tie-2 according to radiological response.

Tie-2 levels prior to treatment according to best overall radiological (RECIST) response. Horizontal lines represent group medians. There was a statistically significant difference between stable disease (SD) and those who showed disease progression.

CR=complete response, PR=partial response.

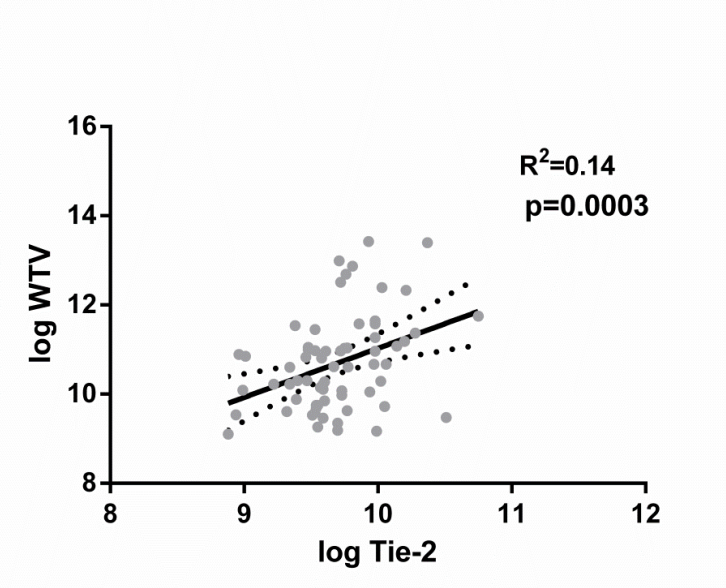

Figure 26: Correlation of Tie-2 levels with tumour volume.

Scatter plot illustrating the positive correlation between log Tie-2 and log whole tumour volume (WTV). The Pearson correlation coefficient (R^2) and significance level are given. Solid line represents the regression, with 95% confidence intervals (dotted).

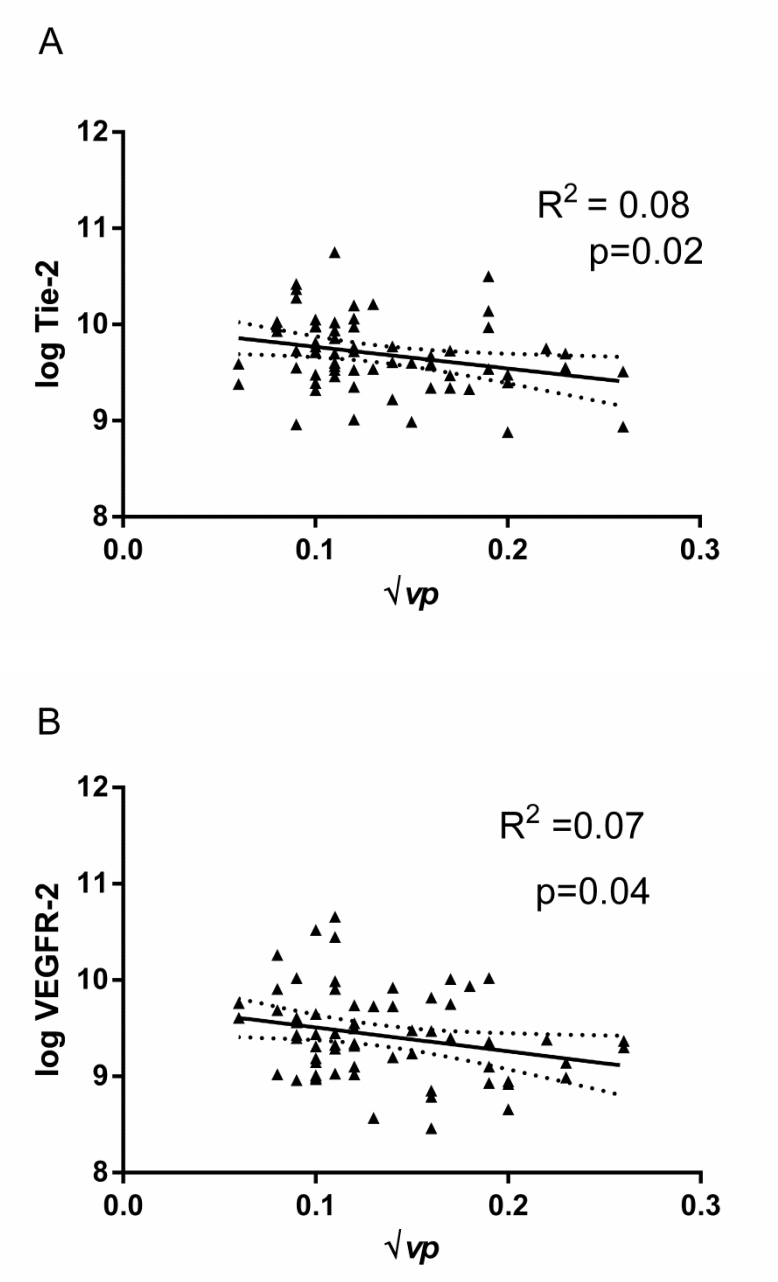

Figure 27: Correlation between tumour blood volume and angiogenic markers

Scatter plots illustrate the weak negative correlations between tumour blood volume (V_p) and (A) Tie-2 or (B) VEGFR-2. The Pearson correlation coefficients (R^2) and significance levels are given. Solid lines represent the regression, with 95% confidence intervals (dotted).

4.3.4 Angiocytokines and clinical factors

Having observed that elevated Tie-2 levels were associated with the development of progressive disease and, that Tie-2 levels were positively correlated with WTV; we wished to test whether Tie-2 might be associated with clinical circulating markers of disease burden, such as CEA, platelet count and serum LDH. We discovered that Tie-2 was significantly correlated with both LDH and CEA (R^2 0.29, p=<0.0001, and R^2 0.12, p=0.0003), respectively (Figures 28 and 29 respectively). However, the strength of these correlations, as indicated by the R^2 value, was weak. No relationship between Tie-2 and platelet count was found. Nor was there evidence of an association between pre-treatment concentrations of VEGF-A, VEGFR-2 or HGF and LDH, CEA or platelet count.

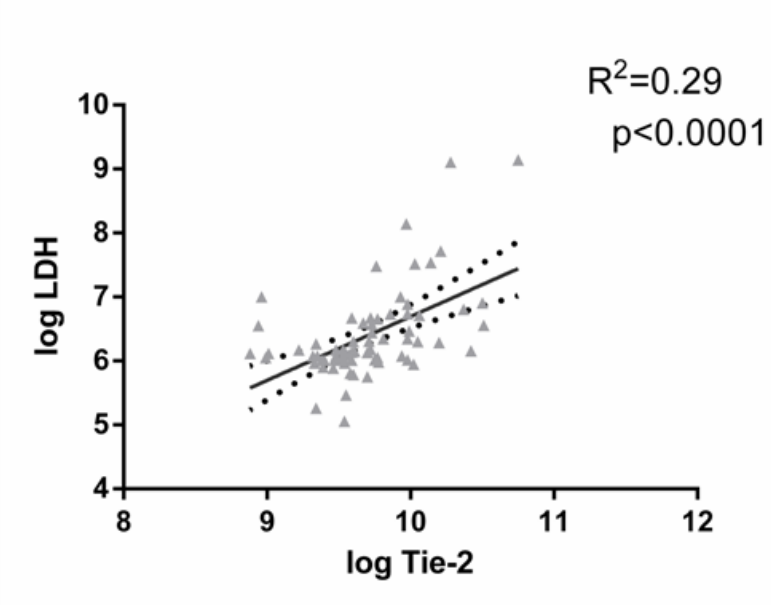

Figure 28: Association between pre-treatment Tie-2 and LDH.

Scatter plot illustrating the positive correlation between log Tie-2 and log serum LDH at baseline. The Pearson correlation coefficient (R^2) and significance level are given. Solid line represents the regression, with 95% confidence intervals (dotted).

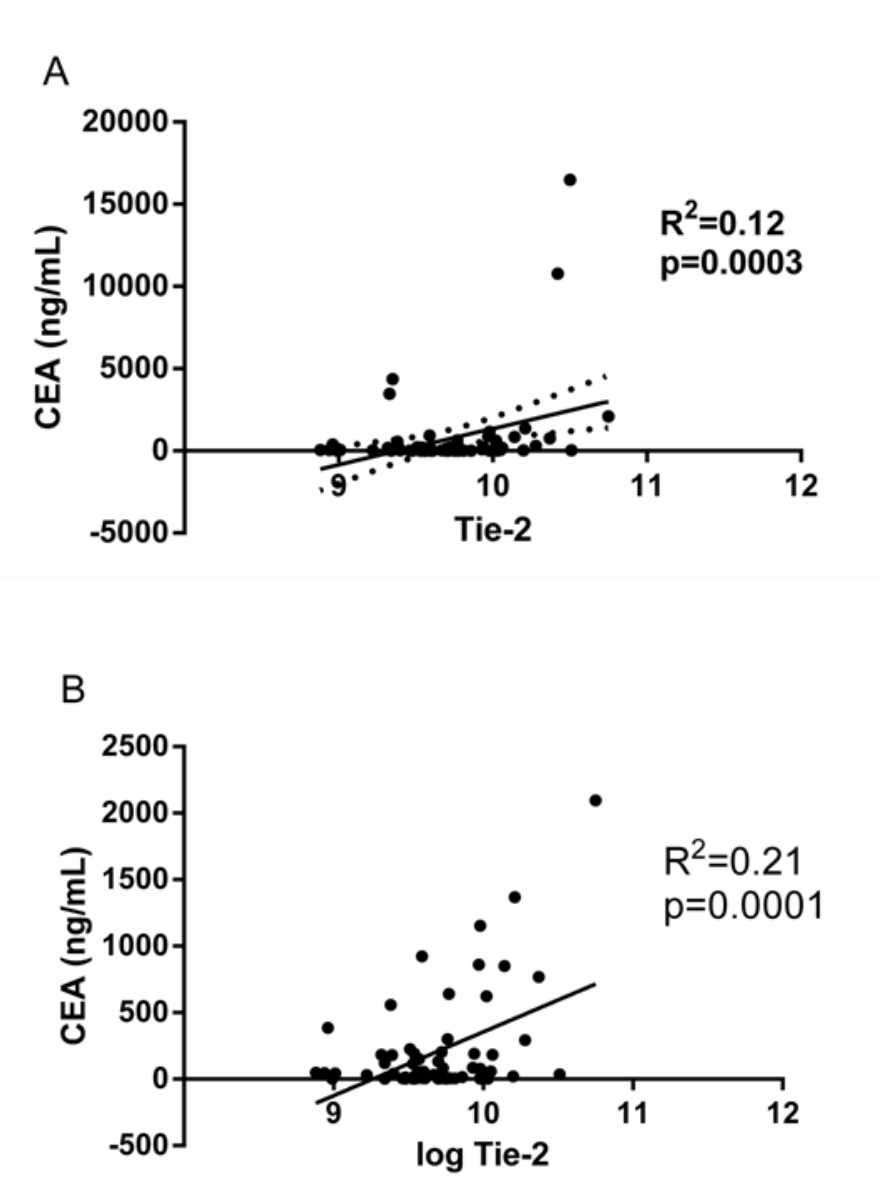

Figure 29: Association between pre-treatment Tie-2 and CEA.

Scatter plots illustrating the positive correlation between log Tie-2 and serum CEA at baseline. Figure A demonstrates that there are four subjects with very high CEA values. Figure B demonstrates that the strength of the correlation is more apparent once these four outliers have been removed. The Pearson correlation coefficients (R^2) and significance levels are given. Solid lines represent the regression, with 95% confidence intervals (dotted).

4.4 Multivariate analysis of baseline variables

Several relevant prognostic factors pre-treatment in patients with metastatic colorectal cancer patients treated with bevacizumab and chemotherapy, were identified as significant from univariate analysis in this chapter. These included LDH, CEA (categorised), performance status, VEGF-A, HGF (categorised) and VEGFR-2 (categorised) and these variable were taken forward for inclusion in a multivariate analysis model. Given the size of our trial, it was not appropriate to include more than 8 variables for inclusion in this model. Categorised K^{trans} was selected on the basis that this was one of the most significant DCE-MRI variables on univariate analysis and because a prognostic role for K^{trans} was a pre-specified hypothesis prior to the study. The inclusion of platelet count in the model reflected the importance of this measurement as an additional prognostic factor in colorectal cancer.

A summary of significant prognostic factors and results according to univariate and multivariate analysis are shown in Table 27. Three variables retained significance on multivariate analysis: performance status (p=0.008), K^{trans} (p=0.015) and HGF (p=0.003).

Variable	Progression Free survival (PFS)			
	Univariate		Multivariate	
	HR(95% CI)	p-value	HR(95% CI)	p-value
Clinical				
LDH**	1.7(1.2-2.4)	**p=0.005**	1.3 (0.9-1.9)	p=0.131
Platelet count **	1.9 (1.0-3.8)	p=0.070	1.0 (0.4-2.4)	p=0.975
CEA * (low vs high)	0.6 (0.3-1.0)	**p=0.037**	0.7 (0.4-1.3)	p=0.250
Performance status (0 vs 1/2)	0.5 (0.3-0.8)	**p=0.004**	0.5 (0.3-0.8)	**p=0.008**
DCE-MRI				
K^{trans}* (low vs high)	1.6 (1.0-2.7)	p=0.065	2.0 (1.1-3.4)	**p=0.015**
Angiocytokine				
VEGF-A**	1.6 (1.1-2.4)	**p=0.020**	1.1 (0.6-2.1)	p=0.640
HGF* (low vs high)	0.5 (0.3-0.8)	p=0.050	0.4 (0.2-0.7)	**p=0.003**
VEGFR-2 * (low vs high)	0.5 (0.3-0.9)	**p=0.020**	0.7 (0.4-1.4)	p=0.300

Table 27: Summary of significant prognostic factors from univariate and multivariate analysis.

Summary of significant prognostic factors from univariate analysis in patients from the Travastin-1 study. *indicates where continuous variables have been dichotomised according to the median value. Hazard ratio (HR) <1.0 favours the low group (or lower values). **indicates continuous variables, here a HR>1.0 indicates a worse outcome for higher values of the variable. Bold type has been used to highlight the significant results from multivariate analysis.

4.4 Discussion

As part of this thesis the reproducibility of DCE-MRI and DWI was assessed in this population of patients with metastatic colorectal cancer. The reproducibility of DWI in a comparable population of 19 patients with colorectal liver metastases was recently reported (Heijmen, Ter Voert et al. 2013). The mean ADC Value in this population was 1.17 x 10^{-3} mm^2/s which was comparable, although slightly higher than our population mean of 1.07 x 10^{-3} mm^2/s. The co-efficient of reproducibility based on the assessment of 21 metastases was 0.20 x10^{-3} mm^2/s which was lower than the co-efficient of reproducibility of 0.34 x 10^{-3} mm^2/s calculated for this study. However, calculations for this study were based on a much larger sample size including 111 lesions in 70 patients and it could be argued that our study better reflects underlying variability in the population. The reproducibility values for the DCE-MRI parameters included the within-patient coefficient of variation were all acceptable and comparable with that from the published literature (Galbraith, Lodge et al. 2002; O'Connor, Carano et al. 2009).

Three-dimensional tumour volume acquisition has been shown to be a sensitive index of response and survival in a localized disease, such as pleural mesothelioma (Ak, Metintas et al. 2009). However, it seems likely that this measure of disease volume under represents the total disease volume for our patients, many of whom had other metastases out-with the field of view of the MR scan. This could explain why baseline WTV failed to emerge as a prognostic factor for PFS or radiological response. Despite this limitation of the assessment of WTV in our population, since the tumour of interest identified on MR corresponded to the largest tumour metastases it would be reasonable to accept WTV as a surrogate for total disease burden. A high correlation between serum LDH and WTV was observed in our patient population; which was absent between CEA or platelet count and WTV.

This finding is consistent with results from the CONFIRM trial in metastatic colorectal cancer (Koukourakis, Giatromanolaki et al. 2011) which found serum LDH was highly correlated with tumour burden and an important prognostic factor.

A negative correlation was observed between K^{trans} and WTV (r-0.37, p=0.002) which is significant given the bias towards large disease volume in our population. This could have resulted in lower than expected values of K^{trans} in our population.

We also found the parameters IAUC-60 and K^{trans} to be very highly correlated in our trial population. This was anticipated since both are composite parameters representing capillary surface area, permeability and blood flow, although IAUC-60 is also affected by Ve. Both have been recommended as appropriate end points for trials (Leach, Brindle et al. 2005), although K^{trans} has been widely adopted as an end point in drug trials assessing anti-angiogenic therapies, as it is felt to be more specific for the assessment of vascular permeability (O'Connor, Jackson et al. 2012). However, values of IAUC-60 were more reproducible than K^{trans} in our population which is at odds with previous reports from our group (Roberts, Issa et al. 2006). Although, an acknowledged limitation of the previous report was the absence of a calculated AIF for each individual, due to acquisition problems; which could account for the discrepancy between results.

Patients who had a radiological response to treatment were more likely to have high values of K^{trans} and IAUC-60 before treatment, although this was only significant for median values of IAUC-60 between patients who achieved a complete or partial response compared with those with stable disease (p=0.0006). Unexpectedly, we also identified a statistically significant difference in parameters Ve, T1 and V_p in patients who achieved a radiological response to treatment versus those with stable disease. Responding tumours could be characterised as more vascular (high IAUC-60, K^{trans} and V_p) and with a larger interstitial space (reflected in the higher values of V_e).

In theory, a tumour micro-environment with these properties should be favourable for the delivery of chemotherapy which may explain the observed improvement in radiological response for a subgroup of patients.

A novel finding from this piece of research was the identification and measurement of significant differences in heterogeneity between subjects and within subjects bearing multiple liver metastases. Both difference measures were highly significant (Chi-squared test, $p<10^{-6}$). To our knowledge, this is the first report of DCE-MRI and DWI biomarkers combined in this way to characterize phenotypic differences in metastases. Few studies have described the heterogeneity between different metastases in a single organ within an individual, although a high concordance between mutations in liver metastases within the same patient has been reported (Jones, Chen et al. 2008).

The growing evidence that significant biological variation exists within and between metastatic deposits implies that heterogeneity of tumour response to different therapies might be predicted if appropriate biomarkers can be developed. This analysis examined these problems using repeated baseline scans to identify and correct for non-biological variation in biomarkers. Furthermore a statistical framework was constructed to allow combination of these corrected variables to increase statistical power. The results demonstrate that conventional DCE-MRI and DWI metrics have sufficient information content to support identification of biological variation in metastatic deposits within and between patients.

The assessment of circulating angiocytokines prior to treatment in relation to outcome was an important aim of this thesis. Pre-treatment levels of VEGF-A were significantly associated with progression free survival on univariate analysis ($p=0.02$), with survival favouring lower values of VEGF-A.

This finding is consistent with reports from large trials in patients with colorectal cancer treated with anti-angiogenic therapy (Hegde, Jubb et al. 2012; Jurgensmeier, Schmoll et al. 2013).

Dichotomised pre-treatment levels sVEGFR-2 and HGF were also significantly associated with progression free survival according to univariate analysis in our patient cohort. However, when all three angiocytokines were included in multivariate analysis, only dichotomised HGF retained significance (p=0.003). There are limited studies evaluating the role of serum HGF in metastatic colorectal cancer. Serum HGF levels were found to be higher in patients with colorectal cancer compared with controls (Fukumura, Xavier et al. 1998). Elevated levels of HGF preoperatively were also associated with disease relapse in patients with liver metastases from colorectal cancer (Osada, Matsui et al. 2010). This study is the first to report that HGF levels were prognostic for outcome in patients with colorectal cancer treated with chemotherapy and bevacizumab and further investigation of this angiocytokine is warranted in future studies.

A number of important observations from our results were made in relation to Tie-2. These included an association between high pre-treatment levels of Tie-2 and the early development of progressive disease, as well as a strong correlation between Tie-2 and whole tumour volume measured by DCE-MRI. Interestingly, Tie-2 was also highly correlated with LDH and CEA which were other markers of disease burden in our population.

The discovery that higher levels of Tie-2 were associated with the development of early progressive disease is consistent with the theory that VEGF-A/VEGFR-2 inhibition can result in up-regulation of alternative signalling pathways, such as the Ang-1, Ang-2/Tie-2 pathway (Bottsford-Miller, Coleman et al. 2012).

Given that our findings were consistent with an association between Tie-2 and tumour burden it is surprising that there appeared to be no apparent relationship to progression free survival. Our findings suggest a role for further evaluation of Tie-2 as an early indicator of disease progression.

An important aim of our study was the comparison of imaging derived biomarkers and angiocytokines as we believed that these two assessments should give complementary information regarding underlying biological mechanisms. Comparison of the pre-treatment angiocytokines and DCE-MRI data identified that there was a weak negative correlation between both Tie-2 and VGFER-2 and the DCE-MRI parameter *Vp*. These findings are consistent with an earlier study from our group which also identified a negative correlation between V_p *and* VEGFR-2 in patients with ovarian cancer following chemotherapy (Mitchell, O'Connor et al. 2010). These results are biologically plausible in that a tumour with a low V_p (which is representative of plasma volume) might be expected to be relatively more hypoxic; which would result in HIF-1 mediated up-regulation of hypoxia responsive genes such as VEGF-A and VEGFR-2. This might explain the weak relationship observed between VEGF-A and V_p (p=0.05), which was also identified, although this was borderline for significance.

The observed relationship between Tie-2 and V_p is inexplicable since the Tie-2 receptor is principally expressed on vascular endothelium (Cascone and Heymach 2012) and would therefore be expected to show a positive, rather than a negative, correlation with V_p.

The most important prognostic factors that emerged from multivariate analysis of pre-treatment clinical factors and biomarkers were: performance status, HGF and K^{trans}. The identification of performance status as one of the most important prognostic factors

in colorectal cancer is widely accepted (Kohne, Cunningham et al. 2002; Chibaudel, Bonnetain et al. 2011).

Researchers who investigated patients with renal cancer identified that a high baseline K^{trans} and high *Vp* dichotomised around the median value, predicted a longer progression free survival in patients before treatment with sorafenib (Hahn, Yang et al. 2008).

However, the prognostic role for HGF and K^{trans} must be viewed as preliminary in this population, particularly given the high correlations between different circulating angiocytokines and between different DCE-MRI biomarkers. Further investigation of the relationship between these biomarkers pre-treatment and outcome is warranted and future work will involved a more detailed statistical analysis of these biomarkers in this context.

The following thesis chapter will investigate dynamic changes in imaging parameters and angiocytokines following treatment with bevacizumab.

Chapter 5: Changes in imaging and circulating biomarkers following bevacizumab

This chapter reports the dynamic changes in circulating angiocytokines and imaging biomarkers in trial subjects following treatment with a single dose of bevacizumab. The methods to acquire this data, the patient population and analysis methods have been previously reported in earlier chapters of this thesis.

5.1 Dynamic changes in DCE-MRI and DWI following bevacizumab

A summary of the values for each of the imaging parameters pre-treatment (baseline), 2 days and 14 days after treatment with a single dose of bevacizumab are given in Table 28. There was a significant reduction in whole tumour volume 14 days after treatment with bevacizumab ($p<0.0001$, Freidman's test) compared to pre-treatment values and day 2 values, as shown in Figure 30A. Likewise, values of T1 were significantly lower at day 14 compared to values pre-treatment and at day 2 ($p<0.0001$, Freidmann's test), as shown in Figure 30G. The DCE-MRI parameters IAUC-60, $K^{trans,}$ V_e, V_p and EF all showed a statistically significant reduction at day 2 which were sustained at day 14 (Figures 30B-F). Diffusion weighted imaging demonstrated that the ADC values increased at day 2 ($p<0.001$, Freidman's test), relative to pre-treatment values but had reduced again by day 14 (Figure 30G).

Imaging Parameter	Baseline		Day 2		Day 14	
	Median (range)	N	Median (range)	N	Median (range)	N
WTV (mm^3)	40,946 (7228-675,494)	70	41,894 (6318-679,443)	65	35,335** (5643-750,183)	67
EF (%)	97.5 (82.0-100.0)	69	93.4** (58.2-100.0)	62	94.0** (0.0-100.0)	64
IAUC ($mmol.min^{-1}$)	16.6 (7.6-35.0)	68	12.2** (3.4-30.0)	62	10.7** (3.7-25.6)	63
K^{trans} (min^{-1})	0.16 (0.06-0.58)	65	0.10** (0.00-0.45)	62	0.10** (0.25-0.36)	63
V_e	0.29 (0.10-0.76)	68	0.26** (0.00-0.49)	62	0.26** (0.10-0.64)	63
V_p	0.014 (0.003-0.068)	68	0.010** (0.003-0.046)	62	0.012** (0.001-0.060)	63
T1 (ms)	1058 (744-1379)	70	1032 (528-1486)	62	974** (526-1748)	63
ADC (mm^2/s)	0.0010 (0.0007-0.0017)	64	0.0010* (0.0007-0.0011)	61	0.0010 (0.0006-0.0017)	62

Table 28: imaging parameters pre-treatment and 2 and 14 days after a single dose of bevacizumab

Data are presented as median (range). N refers to number of evaluable scans at a given timepoint for each parameter.

**p<0.0001 vs baseline scan, *p<0.001 vs baseline scan

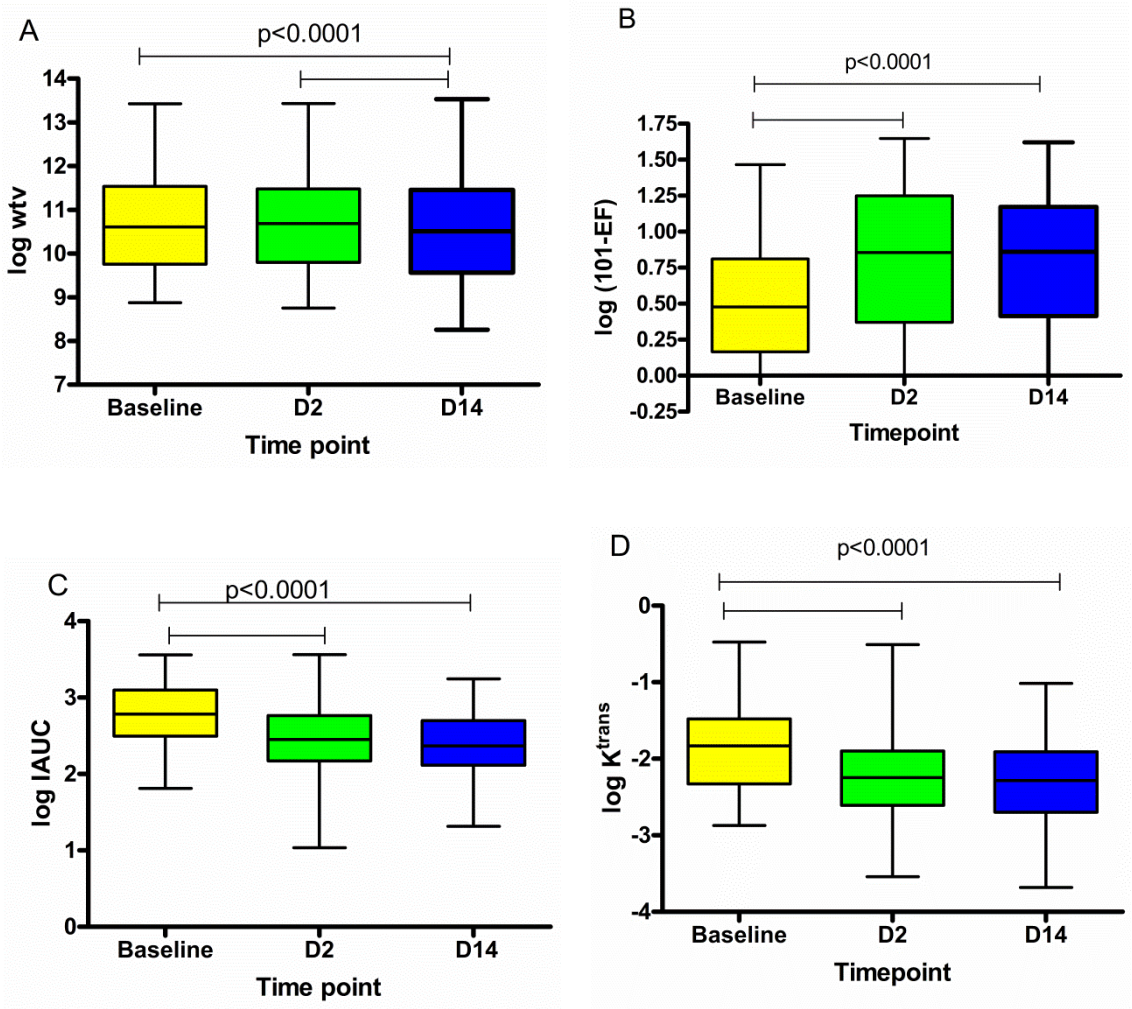

Figure 30: Dynamic changes in imaging parameters at day 2 and 14 post treatment.

A. Whole tumour volume
B. Enhancing Fraction
C. IAUC-60
D. K^{trans}

Log transformed values of each parameter were used for statistical calculations, an inverse log transformation for EF was used: (log (101-EF))

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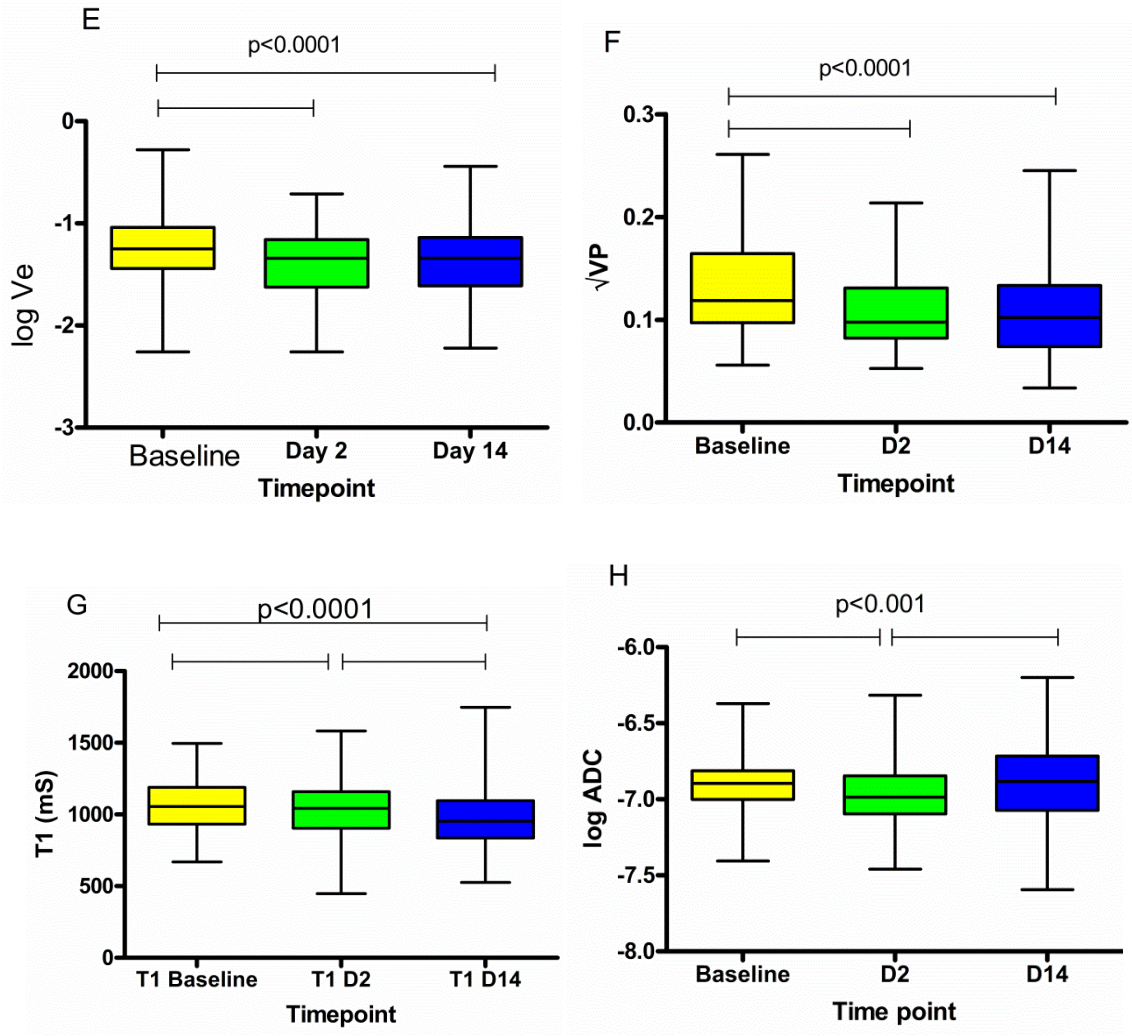


Figure 1: Dynamic changes in imaging parameters at 2 days and 14 days

E. V_e
F. V_p
G. T1
H. ADC

Log transformed values of ADC and Ve were used for statistical calculations and square root transformation of V_p, as detailed in earlier sections of this thesis.

5.1.1 Dynamic changes in MR parameters and relationship with outcome

5.1.1.1 Dynamic changes in DCE-MRI parameters and progression free survival

The magnitude of change for each parameter was calculated from pre-treatment values for each time point for individual subjects. These results were analysed for any association with PFS using a univariate cox regression method and the results are shown overleaf in Table 29.

There was a significant association between the magnitude of change in Enhancing Fraction (EF) 2 days after administration of bevacizumab and progression free survival HR 0.6 (p=0.02). Lower values of the log ratio of enhancing fraction at day 2 favoured a longer progression free survival. There was no association between the magnitude of change from baseline in parameters (WTV, IAUC-60, K^{trans}, V_e, V_p, T1 and ADC) with progression free survival.

Parameter	Baseline-Day 2		Baseline-Day 14	
	HR (95%CI)	*p*	**HR (95%CI)**	*p*
WTV	HR 0.8 (0.2-3.6)	0.75	HR 2.2 (0.7 - 6.7)	0.17
EF	HR 0.6 (0.4-0.9)	**0.02**	HR 0.9 (0.6-1.3)	0.44
IAUC	HR 0.9 (0.3- 2.7)	0.92	HR 0.9 (0.3-2.8)	0.90
K^{trans}	HR 1.0 (0.4- 2.3)	0.98	HR 0.7 (0.4- 1.5)	0.37
V_e	HR 1.6 (0.5- 5.4)	0.44	HR 0.6 (0.2- 1.8)	0.38
V_p*	HR 1.0 (1.0)	0.39	HR 1.0 (1.0)	0.23
T1*	HR 1.0 (1.0)	0.62	HR 1.0 (1.0)	0.79
ADC	HR 0.7 (0.2-3.0)	0.62	HR 1.2 (0.5-3.2)	0.66

Table 29: Association between PFS and biomarker changes from baseline to 2 and 14 days following bevacizumab

Hazard ratio (HR) <1.0 indicates that changes in biomarker values were associated with an improvement in PFS. Log ratios were used to calculate statistical difference in change from baseline, where specified * percentage change from baseline was used.

5.1.1.2 Is the direction of change from baseline in imaging parameters after bevacizumab associated with PFS?

To assess whether the direction of change from baseline in DCE-MRI or DWI parameters might predict clinical outcome, patients were stratified according to whether there had been an absolute decrease (<=0) or increase in any parameter (>0) between day 2 and baseline or day 14 and baseline. Using Kaplan-Meier estimates and log rank tests, survival difference between the two groups was calculated for each parameter 2 days and 14 days following treatment with bevacizumab. These results are summarised below in Table 30.

Parameter	D2 p-value	D14 p-value	PFS
IAUC	0.82	**0.03**	<=0 PFS 46 weeks > 0 PFS 33 weeks
EF	0.53	0.37	
ADC	0.64	0.79	
T1*	**0.04**	0.81	<=0 PFS 39 weeks >0 PFS 62weeks
V_p*	0.88	0.84	
WTV	0.99	0.24	
K^{trans}	0.80	0.86	
V_e	0.76	0.36	

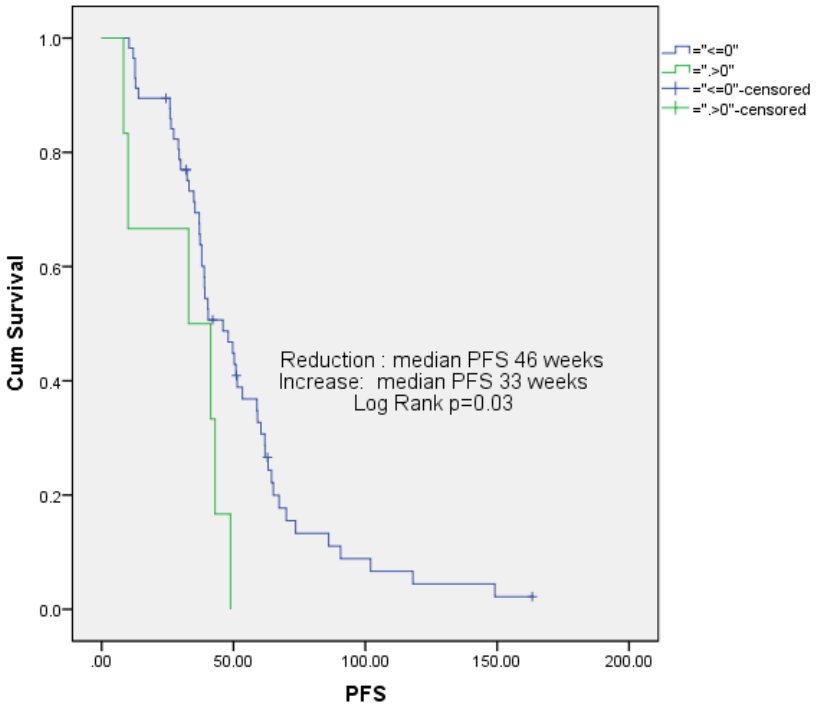
Table 30: Significance of difference in PFS according to directional change in DCE-MRI and DWI-MRI parameters 2 or 14 days following bevacizumab.

Log ratios were used to calculate absolute changes from baseline except where specified * when percentage change was used. PFS is given where difference was significant.

These results demonstrate that parameter change from baseline was significant for IAUC at days 14 (p=0.03) and T1 at day 2 (p=0.04).

Any reduction in IAUC at day 14 following bevacizumab was associated with a prolonged progression free survival compared to patients where there was an absolute increase in IAUC at day 14, 46 weeks v 33 weeks (p=0.03, Log Rank test). In contrast, a relative increase in the value of T1, 2 days following treatment with bevacizumab was associated with a more prolonged progression free survival compared with patients where the value of T1 had reduced from baseline, 62 weeks v 39 weeks (p=0.04, Log Rank test). The Kaplan-Meier survival curves for direction of change from baseline in IAUC at Day 14 and T1 at D2 are shown in Figure 31.

A



B

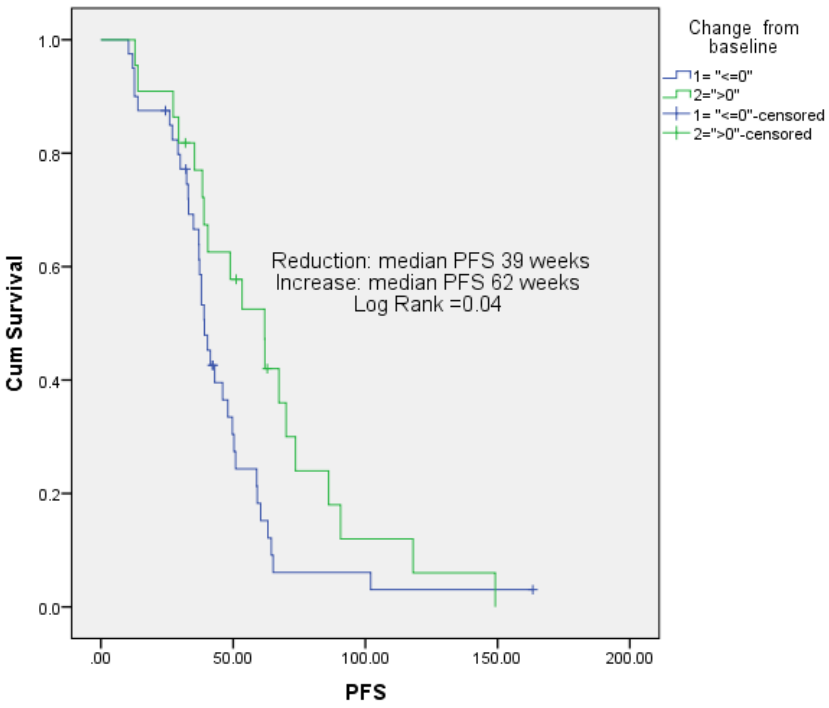


Figure 31: Survival plots based on reduction from baseline in IAUC and T1.

Kaplan-Meier survival plots of progression free survival according to:

(A) Direction of change from baseline in IAUC values at day 14.

(B) Direction of change from baseline in T1 values at day 2.

5.1.1.3 Are median values of imaging parameters after treatment with bevacizumab, associated with radiological response?

The median values of each imaging parameter were compared across subjects at day 2 and day 14 according to the best RECIST response achieved. Results are summarised below in Table 31.

There was a significant difference in median values of IAUC-60 at both day 2 and day 14 according to RECIST response categories (p=0.01 for both, Kruskal Wallis test). At both day 2 and day 14, median values of IAUC-60 were higher for patients who had a complete or partial response, compared to those with stable disease or progression of disease. Scatter plots illustrating changes for median IAUC according to best RECIST response are given in Figure 32A (IAUC at day 2) and 32C (IAUC at day 14). Likewise, higher median values of Ve at day 2 (p=0.02) and higher median values of K^{trans} at day 14 (p=0.03) were observed for the patients who had a complete or partial response compared to the other response categories, as depicted in Figure 32B and 32D.

Median ADC values at day 14 were significantly higher for those subjects who had stable disease compared with other response categories (p=0.01), as shown in Figure 32E. Lower median values of whole tumour volume, were associated with complete or partial response and higher values with disease progression (p=0.03), as shown in Figure 32F. The scatter plot in Figure 32G illustrates that median values of T1 were lower for patients who achieved a complete or partial response compared with other response categories (p=0.02).

Variable	Two sided p-value D2	Two sided p-value D14
IAUC-60	**0.01**	**0.01**
EF	0.14	0.13
ADC	0.06	**0.01**
T1*	0.33	**0.02**
Vp*	0.15	0.08
WTV	0.15	**0.03**
K^{trans}	0.15	**0.03**
Ve	**0.02**	0.05

Table 31: Comparison in median values of imaging parameters according to best RECIST response.

Log transformed variables were used for statistical purposes unless indicated by *.

P values <0.05 were considered significant and are highlighted in bold type.

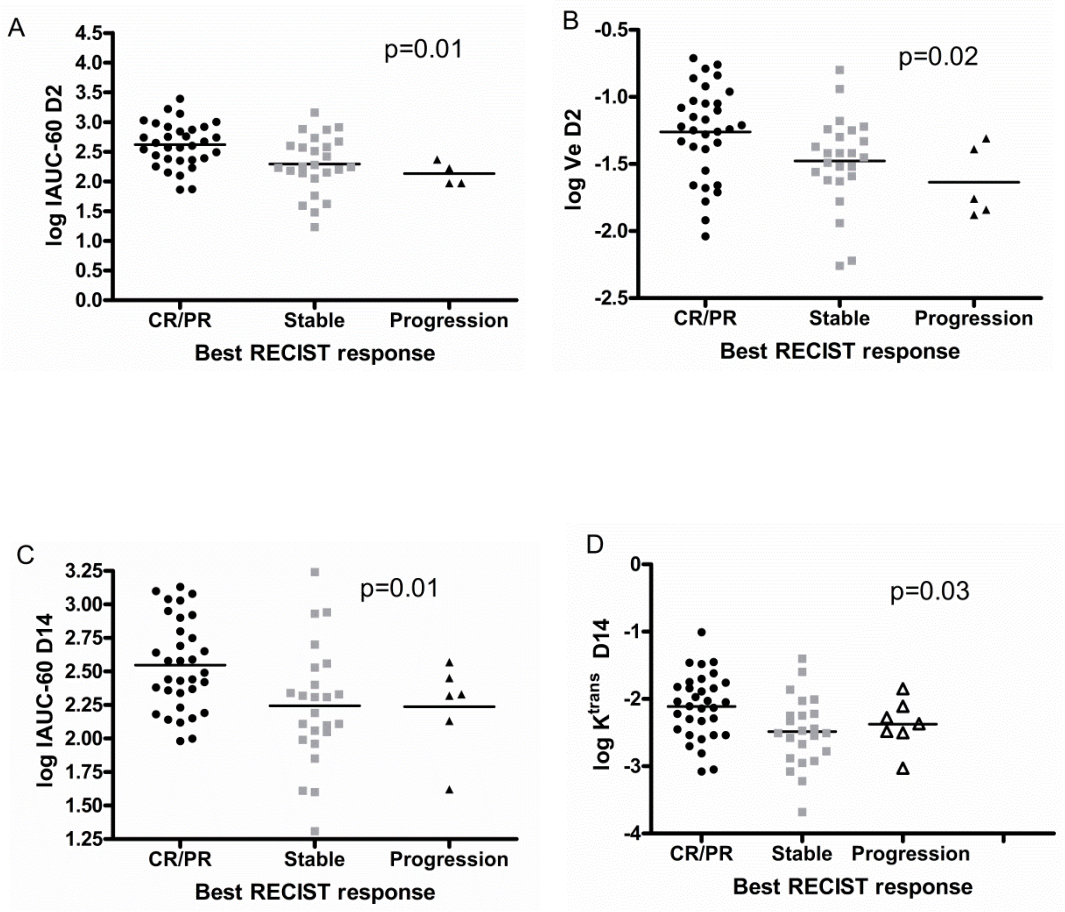


Figure 32: Median parameter values according best RECIST response.

Median values (represented by horizontal bars) were compared across response categories using the Kruskal Wallis test. Each point denotes a separate subject. Log transformed values were used for statistical analysis and all p-values were two sided.

A. IAUC-60 at day 2

B. V_e at day 2

C. IAUC-60 at day 14

D. K^{trans} at day 14

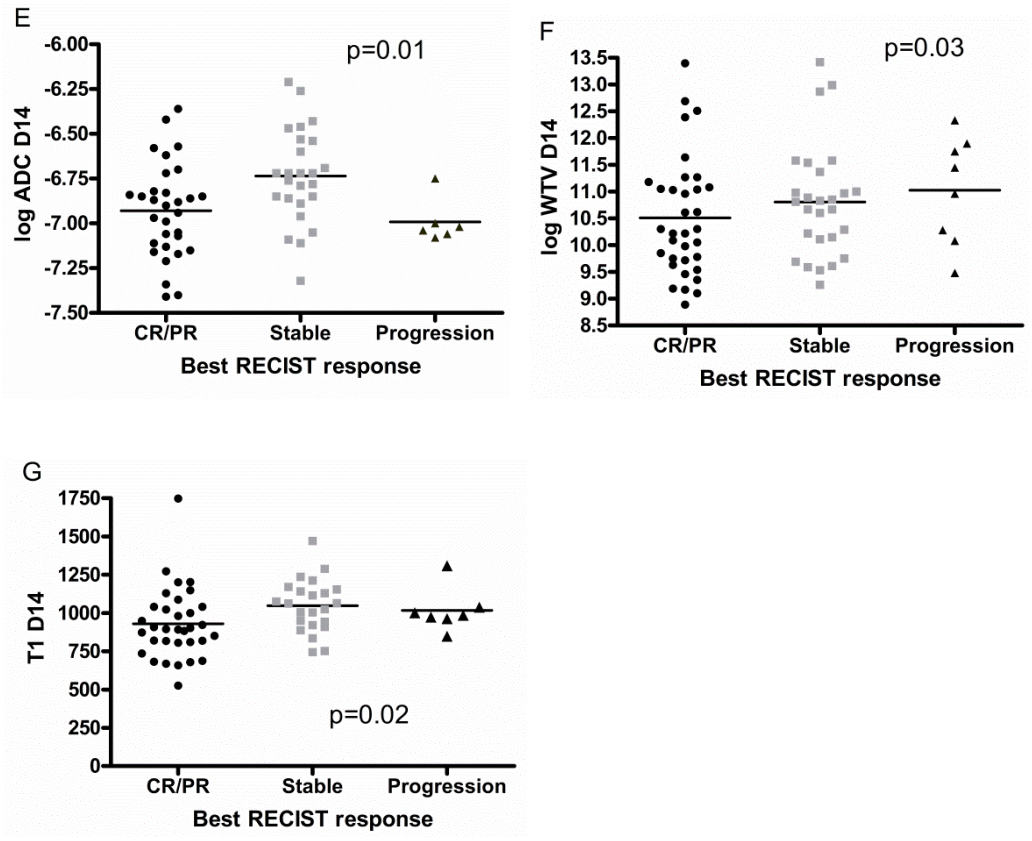


Figure 32 (E-G): Median parameter values according best RECIST response.

Median values (represented by horizontal bars) were compared across response categories using the Kruskal Wallis test. Each point denotes a separate subject. Log transformed values were used for statistical analysis and all p-values were two sided.

E. ADC at day 14

F. WTV at day 14

G. T1 at day 14.

5.2 Dynamic changes in angiocytokines following treatment with bevacizumab

A summary of the values for each angiocytokine prior to treatment (baseline), at 7 days and 14 days after treatment with a single dose of bevacizumab is shown in Table 32.

There was evidence of a significant reduction in levels of Angiotensin-1 at 7 days ($p<0.0001$, Freidman's test) which was sustained at 14 days after treatment with bevacizumab ($p<0.0001$, Freidman's test). Likewise reductions in serum Tie-2 levels and levels of VEGF-A were observed at 7 days ($p<0.0001$, ANOVA) and reduction was sustained at 14 days ($p<0.0001$, ANOVA). Fourteen days after administration of bevacizumab, there was a significant reduction in median values of E-selectin ($p=0.01$, ANOVA). Box plots illustrating significant changes in angiocytokines are shown in Figure 33.

Angiocytokine	Baseline		Day 7		Day 14	
	Median (IQR)	N	Median (IQR)	N	Median (IQR)	N
plasma Ang-1 (pg/ml)	2530 (1644-4550)	70	2114 (1167-4132)	46	2638 (1771-4046)	49
plasma Ang-2 (pg/ml)	467 (326-890)	66	458** (234-861)	45	399** (243-989)	48
plasma E-selectin (pg/ml)	16355 (11491-21918)	70	14634 (11564-21257)	46	13340* (10814-18923)	49
plasma HGF (pg/ml)	414 (273-524)	66	392 (275-755)	45	366 (237-597)	48
plasma sTie-2 (pg/ml)	15583 (13115-21509)	70	14422** (11619-18731)	46	12876** (11364-17101)	49
plasma VEGF-A (pg/ml)	186 (116-287)	70	122** (75-246)	46	132** (78-243)	49
plasma VEGF-D (pg/ml)	608 (283-2446)	64	890 (394-2824)	42	923 (356-2764)	45
plasma VEGFR-2 (pg/ml)	11665 (8919-14984)	67	11840 (9633-15933	43	12614 (9761-16167)	46

Table 32: Angiocytokine levels prior at baseline and 7 and 14 days after a single dose of bevacizumab

Data are presented as median (inter-quartile range). N refers to number of scans assessed at a given timepoint.

*p=0.01 and **p<0.0001 vs baseline scan

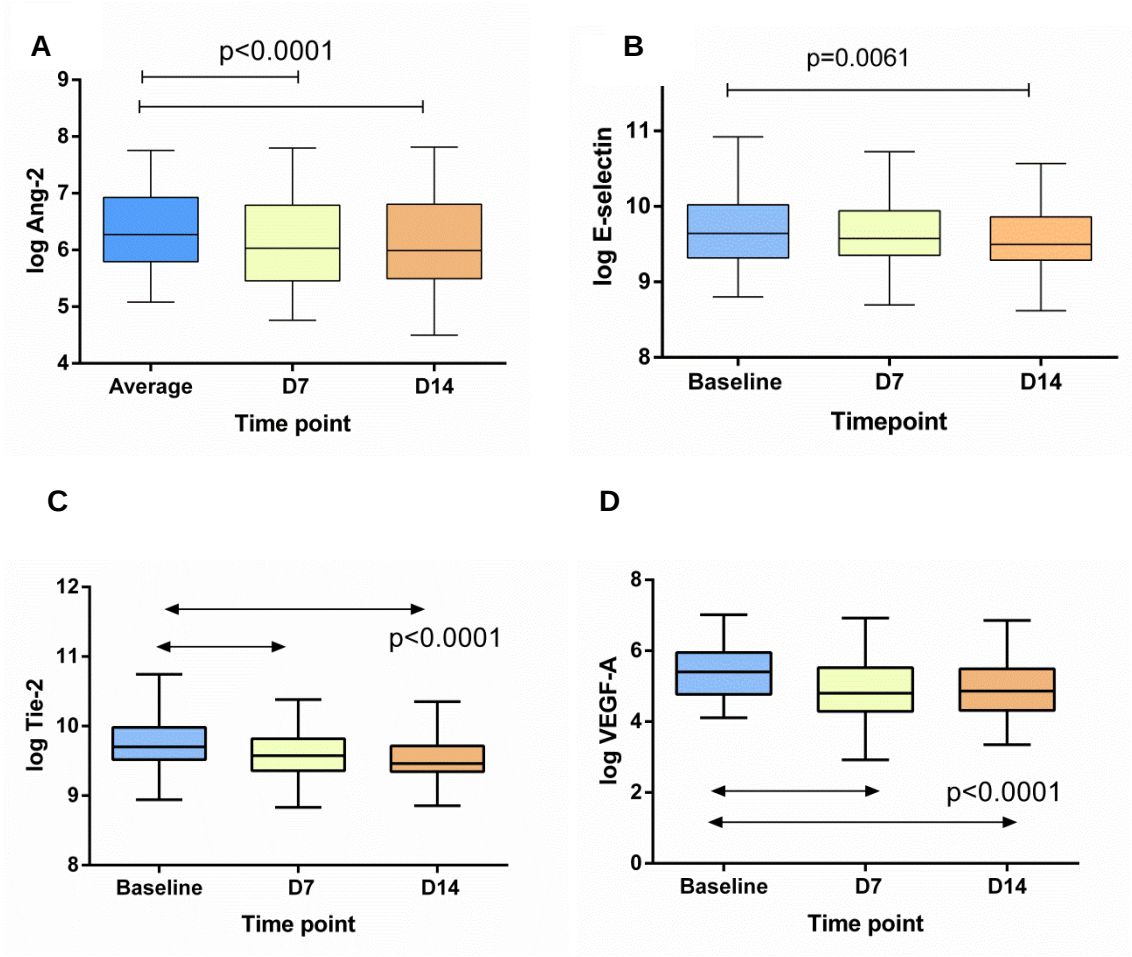


Figure 33: Dynamic changes in circulating angiocytokines relative to baseline at 7 days and 14 days.

Median values of log transformed data were compared across the 3 time points using Freidman's test or ANOVA. Two sided p value <0.05 defined significance.

A. Angiotensin-2
B. E-selectin
C. Tie-2
D. VEGF-A

5.2.1 Dynamic changes in angiocytokines and relationship with outcome

5.2.1.1 Association between dynamic changes in angiocytokines and progression free survival

Changes in levels of angiocytokines at 7 days and 14 days after bevacizumab were analysed for any association with PFS using a univariate cox regression method. The results are summarised below in Table 33. Reduction in levels of VEGF-A 14 days following treatment with bevacizumab was significantly associated with a longer PFS (P=0.04).

Angiocytokine	Baseline-Day 7		Baseline-Day 14	
	HR (95%CI)	***P***	**HR (95%CI)**	***P***
Ang-1	HR 1.0 (0.8-1.3)	0.9	HR 1.1 (0.8 –1.6)	0.3
Ang-2	HR 0.4 (0.1-2.0)	0.3	HR 0.8 (0.2-2.3)	0.6
E-selectin	HR 1.0 (0.4- 33.2)	1.0	HR 1.0 (0.5-18.5)	1.0
HGF	HR 0.5 (0.1- 2.2)	0.4	HR 0.8 (0.2-3.2)	0.8
sTie-2	HR 0.4 (0.8- 2.2)	0.3	HR 0.4 (0.1- 1.5)	0.2
VEGF-A	HR 0.8 (0.6-1.0)	0.1	HR 0.5 (0.2-1.0)	**0.04**
VEGF-D	HR 0.6 (0.3-1.2)	0.1	HR 0.6 (0.3-1.3)	0.2
VEGFR-2	HR 0.3 (0.0-2.1)	0.2	HR 0.6 (0.8-4.5)	0.6

Table 33: Association between PFS and dynamic changes in levels of angiocytokines after bevacizumab

Results of univariate cox regression analyses for levels of angiocytokines at 7 days and 14 days following bevacizumab. Hazard ratio (HR) <1.0 indicates that reductions in the log ratio of the biomarker were associated with a longer PFS. Significant p values were defined as <0.05 and highlighted in bold type.

5.2.1.2 Is direction of change from baseline in levels of angiocytokines associated with PFS following bevacizumab?

To assess whether the change from baseline in levels of angiocytokines might predict clinical outcome, patients were stratified according to whether there had been an absolute decrease or increase in levels between day 7 and baseline or day 14 and baseline (<=0 v >0). Using Kaplan-Meier estimates and log rank tests, difference between the groups was calculated for each parameter 7 days and 14 days following treatment with bevacizumab. There was no significant difference in PFS for any of the angiocytokines investigated according to absolute increase or decrease from baseline at day 7 or day 14. These results are summarised below in Table 34.

Angiocytokine	D7 p-value	D14 p-value
Ang-1	0.22	0.54
Ang-2	0.8	0.7
E-selectin	0.2	0.7
HGF	0.6	0.3
sTie-2	1.0	0.4
VEGF-A	0.1	0.4
VEGF-D	0.1	0.9
VEGFR-2	1.0	0.5

Table 34: Difference in PFS according to reduction or increase from baseline in levels of angiocytokines at 7 or 14 days post bevacizumab.

Log ratios were used to calculate absolute changes from baseline except where specified * when percentage change was used.

5.2.1.3: Dynamic changes in angiocytokines and radiological response

The median values of angiocytokines were compared at day 7 and day 14 according to best RECIST response achieved for each subject. Patients who developed early progression of disease had higher median levels of Tie-2 at 7 days following treatment with bevacizumab, compared with patients in other response categories as demonstrated in Figure 34. There was no association between median levels of Ang-1, Ang-2, VEGF-A, VEGF-D, VEGFR-2, HGF or E-selectin and best RECIST response.

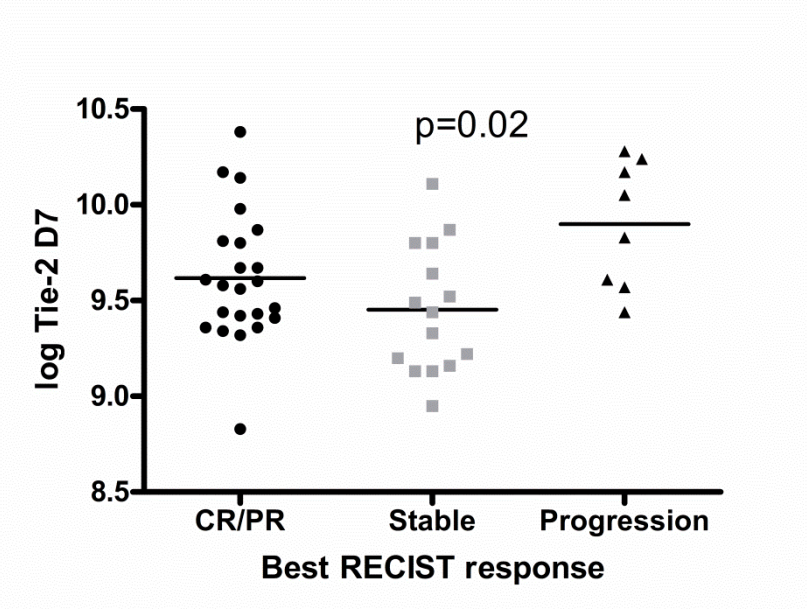


Figure 34: Tie-2 levels at day 7 according to best RECIST response

Median values (represented by horizontal bars) were compared across response categories using the Kruskal Wallis test. Each point denotes a separate subject. Log transformed values were used for statistical analysis and p-values were two sided.

5.3 Correlations between imaging and circulating biomarkers following administration of bevacizumab

5.3.1 Whole tumour volume correlated with sTie-2

There was a significant correlation between values of WTV at 2 days and 14 days with levels of Tie-2 at 7 days and 14 days. Tie-2 levels at day 7 were weakly correlated with tumour volume at day 2, r=0.31(p=0.03 Pearson's test) and at day 14, r=0.31 (p=0.04 Pearson's test). Similarly, Tie-2 levels at day 14 were weakly correlated with tumour volume at day 2, r=0.34 (p=0.02 Pearson's test) and day 14, r=0.32 (p=0.04 Pearson's test). There was no correlation between the log ratio of change from baseline between Tie-2 at day 7 or 14 and WTV at day 2 or day 14. There was no correlation between levels of VEGF-A, VEGF-D, VEGFR-2, Ang-1, Ang-2, E-selectin or HGF at days 7 or 14 and whole tumour volume at day 2 or 14. Figure 35 illustrates correlations between levels of Tie-2 14 days after bevacizumab treatment and whole tumour volume.

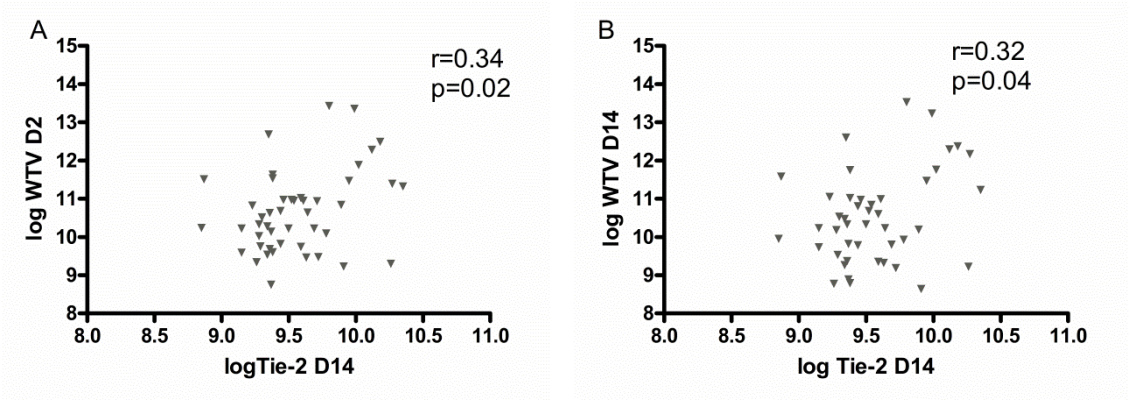


Figure 35: Correlation between Tie-2 levels at day 14 and tumour volume

Scatter plot illustrating the positive correlation between Tie-2 and whole tumour volume at day 2 (A) and day 14 (B). The Pearson correlation co-efficient (r) and significance level (p) are given.

5.3.2 IAUC-60 correlated with Ang-1

Values of IAUC-60 at day 14 correlated with Ang-1 levels at day 14, r=-0.32 (p=0.03, Pearson's test). A scatter plot of the relationship is shown in Figure 36. There was no correlation between levels of VEGF-A, VEGF-D, VEGFR-2, sTie-2, Ang-2, E-selectin or HGF at days 7 or 14 and IAUC-60 at day 2 or 14. Nor was there a significant correlation between values of IAUC-60 at 2 days and levels of Ang-1 7 days after bevacizumab.

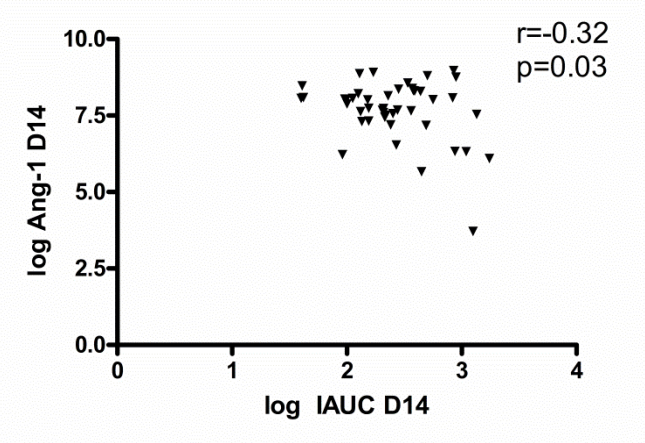


Figure 36: Correlation between levels of Ang-1 and values of IAUC-60 14 days after treatment with bevacizumab.

Scatter plot illustrating a negative correlation between Ang-1and IAUC-60. The Pearson correlation co-efficient (r) and significance level (p) are also given.

5.3.3 V_e correlated with VEGF-A, Ang-1, Ang-2 and HGF

There was no significant correlation between levels of angiocytokines at 7 or 14 days and values of Ve 2 days after treatment with bevacizumab. At 14 days following administration of bevacizumab, values of V_e correlated negatively with VEGF-A (p= 0.01), Ang-1 (p=0.03), Ang-2 (p=0.04) and HGF (p=0.03). There was also a correlation between levels of HGF at 7 days and values of Ve 14 days after bevacizumab. In each case, higher values of angiocytokines were associated with lower values of Ve. Significant correlations are depicted in scatter plots in Figure 37.

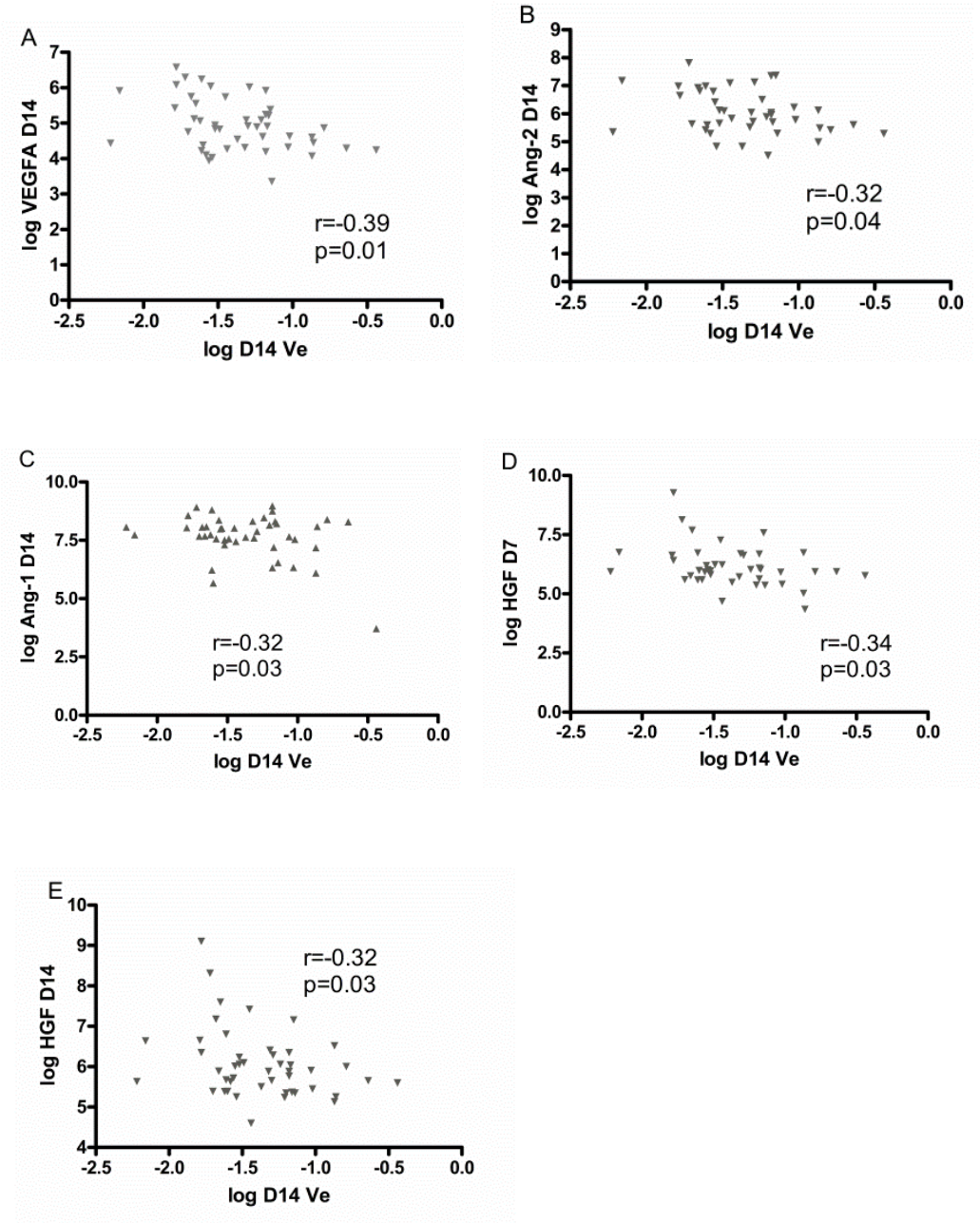


Figure 37: Correlation between values of V_e 14 days after treatment with bevacizumab and circulating angiocytokines.

Scatter plot illustrating negative correlations between V_e and circulating angiocytokines (A) VEGF-A, (B) Ang-2, (C) Ang-1, (D) HGF at 7 Days and (E) HGF at 14 days. The Pearson correlation co-efficient (r) and significance level (p) are given for each graph.

5.3.4 Enhancing Fraction correlated with VEGF-A

At 14 days following treatment with bevacizumab, values of EF weakly correlated with levels of VEGF-A at day 14, r=0.3 (p=0.04, Pearson's test), as shown in Figure 38. There was no correlation between levels of VEGF-D, VEGFR-2, sTie-2, Ang-1, Ang-2, E-selectin or HGF at days 7 or 14 and EF at day 2 or 14. Levels of VEGF-A at 14 days were not correlated with EF 2 days after treatment with bevacizumab.

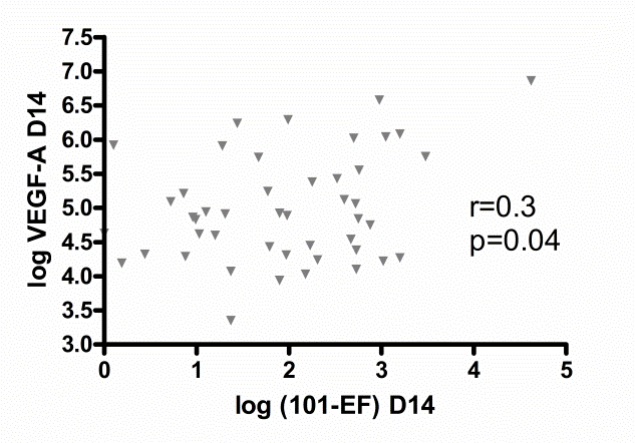


Figure 38: Correlation between values of EF and levels of VEGF-A 14 days after treatment with bevacizumab and circulating angiocytokines.

Scatter plot illustrating a weakly positive correlation between transformed EF and VEGF-A 14 days after bevacizumab. The inverse transformation of EF means that higher values of EF are associated with lower values of VEGF-A. The Pearson correlation co-efficient (r) and significance level (p) are given.

5.3.5 V_p correlated with VEGF-A and VEGFR-2

At 14 days following treatment with bevacizumab, values of Vp correlated weakly with levels of VEGF-A at day 14, r=0.3 (p=0.03, Pearson's test). There was also evidence of a weak negative correlation between levels of VEGFR-2 at day 14, r=-0.3 (p=0.04, Pearson's test). Correlations between V_p at day 14 with VEGFA and VEGFR-2 are shown in Figure 39.

There was no correlation between levels of VEGF-D, sTie-2, Ang-1, Ang-2, E-selectin or HGF at days 7 or 14 and V_p at day 2 or 14. Levels of VEGF-A or VEGFR-2 at 7 days were not correlated with V_p at either 2 days or 14 days after treatment with bevacizumab. There was no correlation between levels of circulating angiocytokines at day 7 or 14 and values of T1 or K^{trans} at 2 days or 14 days after bevacizumab.

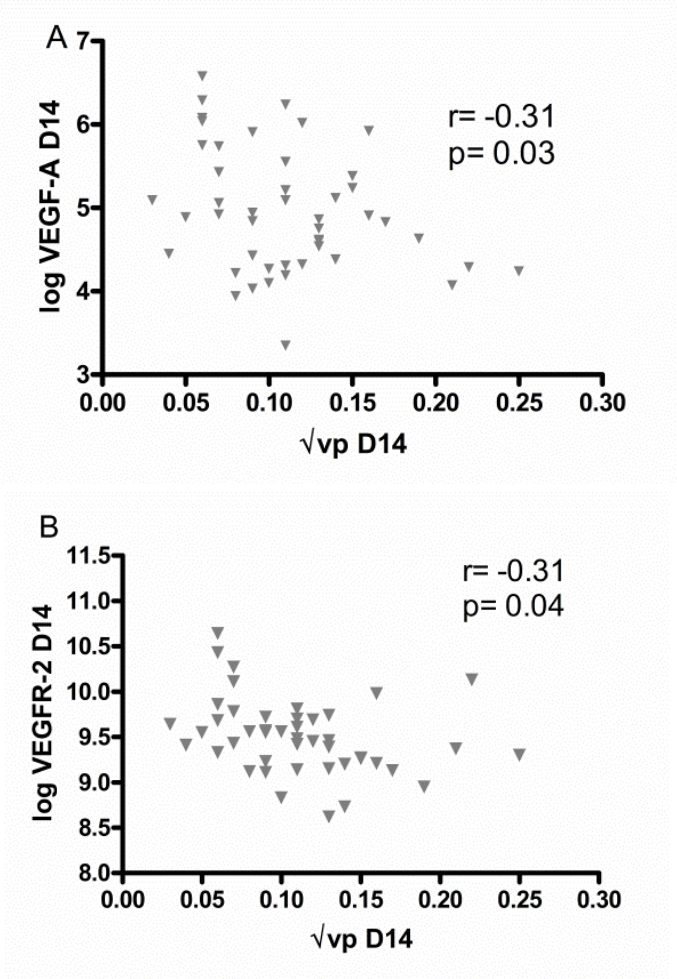


Figure 39: Correlation between values of V_p at day 14 and levels of (A) VEGF-A and (B) VEGFR-2

Scatter plots illustrating (A) positive correlation between VEGF-A and V_p 14 days after bevacizumab and (B) negative correlation between VEGFR-2 and V_p 14 days after bevacizumab. The Pearson correlation co-efficient (r) and significance level (p) are given.

5.4 Discussion

This study builds on an earlier pilot study from our group which investigated temporal changes in DCE-MRI imaging parameters following therapy with bevacizumab, in ten patients with metastatic colorectal cancer (O'Connor, Carano et al. 2009). Consistent with results from the latter study, these data demonstrate an early reduction in enhancing fraction (EF) at 2 days which was sustained until day 14. However in the current study, reductions in the parameters K^{trans}, IAUC-60, V_e and V_p were also significant at 2 days and remained significant at day 14. The time course for the acquisition of imaging data in the pilot study included additional imaging 4 hours and 8 days after bevacizumab. In the pilot study, reductions in V_p, Ktrans and T1 were all significant at 4 hours but not at 2 days or 8 days; although reductions in V_p and T1 were again significant by day 14. The reduction in T1 values and WTV at day 14 is consistent with the larger study and with improvements in tumour associated oedema. It is possible that the pharmacodynamic effects from bevacizumab on the vasculature may be more pronounced at the earlier time point of 4 hours following treatment. Also the sample size in the pilot study may have been insufficient to detect smaller effect changes at the other time points.

A significant advantage of the current study is the larger sample size: 70 patients compared with 10 patients in the original study. This larger study allowed for comparison of dynamic changes in imaging to be correlated with outcome. However, although most of the parameters assessed showed significant change from baseline at either, day 2 or day 14 the magnitude of these changes was not associated with progression free survival, with the exception of the EF. Only the magnitude of change in the enhancing fraction (EF) at 2 days after bevacizumab was associated with progression free survival ($p=0.02$).

The nature of the transformation used for EF in these analyses requires a careful interpretation of the results. Thus the association between a low log ratio value for the transformed negative value of EF (101-EF) and longer PFS indicates that a greater reduction in EF was associated with a longer progression free survival.

The addition of diffusion weighted imaging in the current study provides additional complementary information regarding the changes in the tumour microenvironment following bevacizumab. In contrast to the DCE-MRI parameters, all of which showed a reduction following treatment with bevacizumab, median tumour ADC values were significantly increased 2 days after therapy ($p<0.001$) but this was not sustained at day 14. These results are consistent with findings from both the preclinical and clinical setting and could be related to cell death, necrosis or oedema. Researchers, who treated rats bearing rhabdomyosarcoma with vascular disrupting agents, found that ADC values had increased at 2 days (Thoeny, De Keyzer et al. 2005). Pathologically this was associated with only a small rim of viable tumour tissue.

Likewise in vivo, patients with metastatic renal cancer who received anti-angiogenic therapy with sunitinib showed an increase in tumour values of ADC 3 days after treatment with a return to baseline levels by day 10 (Desar, ter Voert et al. 2011). The outcome was compared between patients categorised according to whether there was evidence of parameter reduction or increase, at 2 days and at 14 days following treatment. Two days after treatment, patients whose tumours showed an absolute increase in T1 values had a significantly longer PFS of 62 weeks versus 39 weeks ($p=0.04$). A significant improvement in PFS was also observed for patients whose tumours showed a reduction in IAUC-60 at 14 days, (46 weeks versus 33 weeks, $p=0.03$).

Our results contrast with those from a previous trial in renal cancer patients treated with sorafenib (Hahn, Yang et al. 2008). These researchers investigated whether the absolute direction of change in DCE-MRI parameters V_p, IAUC and K^{trans} at 4 weeks correlated with PFS and found no significant difference in the outcome between the groups. The results from the current study may be specific to bevacizumab or alternatively, the lack of positive findings in the sorafenib study may relate to the pharmacodynamic assessments at 4 weeks rather than 14 days.

The median values of imaging parameters after treatment with bevacizumab showed a significant association between RECIST response categories. A radiological response to treatment was associated with higher levels of tumour enhancement, lower values of T1 and smaller tumour volumes. In particular, higher median values of IAUC-60 at two days and 14 days were associated with patients who had a good radiological response. These results were consistent with earlier findings which demonstrated that higher median values of IAUC-60 pre-treatment were associated with a radiological response to treatment. Median values of K^{trans} at 14 days were also higher in those patients who achieved a radiological response. At 14 days, patients who subsequently achieved a radiological response had lower median values of T1, consistent with pre-treatment findings. A high median V_e prior to treatment or after 2 days was also associated with a radiological response to treatment. These characteristics might represent an environment which favours a more successful delivery of chemotherapy due to adequate perfusion.

Following treatment with bevacizumab, there was a significant reduction in levels of Ang -2, Tie-2 and VEGF-A by 7 days, which was sustained at day 14. E-selectin levels had also significantly reduced by day 14.

A reduction in VEGF-A levels from baseline to day 14 was significantly associated with a longer progression free survival (HR 0.5, p=0.04).

A consistent observation in this thesis was the relationship between Tie-2 and radiological response. Median levels of Tie-2 pre treatment were higher in the group of patients who developed early progression of disease 7 days after treatment with bevacizumab. Although, overall, there was a significant reduction in levels of Tie-2 at day 7 and day 14 compared with those pre-treatment. A positive correlation was also noted between Tie-2 at day 14 and WTV at both day 2 and day 14, although there was no correlation between dynamic changes in the parameters at either time point. These data are comparable to studies in glioblastoma patients treated with cediranib where a correlation between tumour progression and Tie-2 was observed ((Batchelor, Duda et al. 2010).

Comparison of dynamic changes in circulating and imaging biomarkers in this study was limited by the timing of data acquisition following bevacizumab. Imaging was acquired at day 2 but circulating markers were not collected until day 7, although subsequent imaging time points coincided with the collection of circulating markers. This could explain why the correlations between imaging biomarkers and angiocytokines were observed at day 14. Levels of IAUC60 and Ang-1 at 14 days were weakly negatively correlated such that decreasing levels of IAUC-60 were associated with higher levels of Ang-1. Median levels of Ang-1 had increased by day 14 although this difference was not significant. This could be in keeping with the proposed role of Angiotensin-1 in vascular remodelling and resistance to therapy secondary to VEGF stimulation and hypoxia (Huang, Bae et al. 2009). However, the decrease in Tie-2 levels at day 14 and an absence of any correlation between Tie-2 and IAUC-60 does not support activation of the Tie-2/ Ang-1 signalling pathway in this context.

A novel finding in this study was the evidence of multiple weak negative correlations between Ve and VEGF-A, Ang-2, Ang-1 and HGF 14 days after treatment with bevacizumab. The relationship between V_e and VEGF-A was the most significant (r=-0.39, p=0.01) and suggests that after treatment with bevacizumab, high levels of VEGF-A were associated with a smaller extra-cellular extra vascular space. There was an absence of any relationship between angiocytokines and Ve prior to treatment with bevacizumab. Due to the large number of statistical tests carried out in this study, there is a possibility that false positive results were generated, as no correction was made for multiple comparisons. However, given the exploratory nature of this study it was felt important not to miss potentially significant signals in the knowledge that these could be validated in further studies.

Although levels of EF and VEGF-A appeared to be positively correlated a negative transformation was used for EF which means that back transformed values would be negatively correlated. Thus higher levels of VEGF-A would be associated with lower values of enhancing tumour consistent with chaotic blood supply and necrosis associated in the microenvironment of highly angiogenic tumours.

A weak negative correlation was observed between V_p and VEGFR-2 (r= -0.31, p=0.04) and between Vp and VEGF-A (r= -0.31, p=0.03). This would be consistent with a reduction in tumour blood supply leading to hypoxic stress and increased expression of hypoxia response genes including VEGF and VEGFR-2. These findings are consistent with a study which investigated patients with ovarian cancer treated with chemotherapy, which also reported a negative relationship between VEGFR-2 and V_p (Mitchell, O'Connor et al. 2010).

To sum up, there was a strong pharmacodynamic signal within the imaging markers at the time points selected for this study.

Despite this, the magnitude of change from baseline was only associated with enhancing fraction at 2 days after treatment. However, an absolute reduction in IAUC-60 at 14 days, or increase in T1 at 2 days, favoured a longer progression free survival.

There was a strong association between RECIST response categories and DCE-MRI and DWI biomarkers, particularly at 14 days. The circulating biomarkers Ang-2, VEGF-A and sTie-2 were significantly reduced at 2 days and 14 days. However, only reduction in VEGF-A at 14 days was significantly associated with PFS. Median levels of sTie-2 at 7 days were higher in patients with early disease progression. Tumour volume and sTie-2 levels were positively correlated. A novel finding was the multiple negative correlations observed between V_e and angiocytokines Ang-2, HGF, VEGF-A and Ang-1 at day 14.

These findings represent important additions to our understanding of dynamic changes in response to bevacizumab. The results presented here will be informed by future work from the Travastin-1 trial which will investigate the same biomarkers in relation to chemotherapy plus bevacizumab, in addition to separate studies examining the effect of chemotherapy alone on a panel of angiocytokines and imaging biomarkers in a comparable patient population

6. Discussion and Future Work

A key objective of this thesis was to evaluate, DCE-MRI, DWI and circulating angiocytokines as potential biomarkers in patients with metastatic colorectal cancer recruited to the Travastin-1 trial. As part of this work it was important to establish the clinical outcome in this trial population in relation to known prognostic factors in metastatic colorectal cancer. Of the seventy-six patients who were recruited to the trial, six patients who did not receive treatment were excluded from further analyses. The median age of patients recruited to the trial was 63 years, younger than 70 years which is the median age of diagnosis for patients with metastatic colorectal cancer. Patients also had a good performance status, as only 4% of patients were performance status 2.

An important consideration in our study was the trial inclusion criteria which required patients to have at least one tumour measuring over 3cm. This potentially skewed our population towards an adverse prognostic group with poorer outcomes. However, despite this limitation, the median overall survival in our population of 17.8months (95% CI:14.9-20.9 months) was comparable with that of 19.8 months reported from a recent pooled analysis of seven trials involving patients with metastatic colorectal cancer treated with bevacizumab (Hurwitz, Tebbutt et al. 2013). Furthermore, the median progression free survival of 9.3 months for patients on the Travastin-1 trial (95% CI: 8.5-11.3 months) is consistent with findings from the Saltz study (Saltz, Clarke et al. 2008). Surprisingly, given the large tumour volume for our population, the overall response rate of 44% exceeded the 39% response rate reported in the recent pooled analysis by Hurwitz and colleagues (Hurwitz, Tebbutt et al. 2013).

The clinical prognostic factors investigated using univariate analysis in our population were age, number of disease sites, performance status, CEA, LDH, platelet count, gender and tumour grade. However, only serum LDH, CEA (categorised) and ECOG performance status were significantly associated with PFS. Of these variables, only performance status retained significance on multivariate analyses for PFS (p=0.008).

The reproducibility for DCE-MRI and DWI was assessed in this population of patients. The values obtained were consistent with those from the pilot study carried out by our group in a similar patient population. The within-patient coefficient of variation (wCV) for K^{trans} in this study was 25.7% which was higher than that of 15.4% from the earlier study (O'Connor, Carano et al. 2009). However, the results from the current study are consistent with other reports for the reproducibility of K^{trans} in the literature (Galbraith, Lodge et al. 2002; Morgan, Utting et al. 2006). Our reproducibility values for ADC were acceptable, (wCV) of 12.1%, and consistent with work from other research groups (Heijmen, Ter Voert et al. 2013).

There was a strong association between DCE-MRI parameters and RECIST response categories before and after treatment with bevacizumab. Prior to treatment, the tumours of patients who subsequently had a complete or partial response to treatment had higher median values of IAUC-60, V_e, and V_p, and lower median values of T1. With the exception of V_p, the relationship between median parameter values and RECIST response categories was unaltered after treatment with bevacizumab. Although following treatment with bevacizumab, there were differences between median values of WTV, K^{trans} and ADC; according to RECIST response categories. One possible explanation of these observations is that the DCE-MRI parameters, particularly IAUC-60 identify those tumours which have better perfusion, which in turn favours the effective delivery of cytotoxic therapy.

Alternatively these findings could be the result of correlating two separate radiological investigations assessing the same regions of interest, where both techniques are dependent on adequate contrast enhancement.

The assessment of imaging biomarkers in relation to outcome was an important aim of this thesis. In this study pre-treatment DCE-MRI parameters and DWI parameters did not significantly correlate with PFS on univariate analysis. However, baseline K^{trans} dichotomised according to the median value was a significant variable on multivariate analysis (p=0.015), with a higher value of K^{trans} favouring a longer PFS. Pre-treatment levels of VEGF-A, sVEGFR-2 and HGF were identified as significantly associated with progression free survival on univariate analysis in our patient cohort. For each of these angiocytokines, lower concentrations were associated with a longer progression free survival.

The dynamic changes in imaging biomarkers were assessed after treatment with bevacizumab. A strong pharmacodynamic signal was observed in relation to the imaging parameters at the group level at both 2 days and 14 days after bevacizumab. There was a significant association between a reduction in Enhancing Fraction (EF) 2 days after bevacizumab and PFS (p=0.02). The effect of directional change in a parameter from baseline was also assessed in relation to PFS using Kaplan-Meier estimates. Any reduction in IAUC-60 by day 14 was associated with a longer PFS (46 v 33 weeks, p=0.03). In contrast, any increase in T1 by day 2 was associated with a longer PFS (62 v 39 weeks, p=0.04).

Pre-treatment levels of VEGF-A, sVEGFR-2 and HGF were identified as significantly associated with progression free survival on univariate analysis in our patient cohort. For each of these angiocytokines, lower concentrations were associated with a better progression free survival.

Following treatment with bevacizumab, the strongest pharmacodynamic signals from angiocytokines assessed at the group level were for Ang-2, VEGF-A and sTie-2. For these biomarkers there was a significant reduction in values at both 7 days and 14 days. Values of E-selectin were significantly reduced by day 14. Levels of VEGF-A 14 days after treatment with bevacizumab were significantly associated with PFS on univariate analysis (p=0.04), with lower levels favouring a longer PFS.

An important aim of our study was the comparison of imaging derived biomarkers and angiocytokines as we believed that these different modalities could provide complementary information regarding underlying biological mechanisms. Comparison of the pre-treatment angiocytokines and DCE-MRI data identified a weak negative correlation between VGFER-2 and the DCE-MRI parameter V_p. This correlation was still evident 14 days after treatment with bevacizumab, at which point a negative correlation between V_p and VEGF-A was also noted. Pre-treatment, there was a negative correlation between K^{trans} and WTV as well as between IAUC and WTV.

Significant correlations between Whole Tumour Volume (WTV) and serum Tie-2 levels were observed before and after treatment with bevacizumab. Median serum levels of Tie-2 were higher before and after treatment with bevacizumab in patients who developed early progression of their disease. Fourteen days after treatment with bevacizumab, Ang-1 and IAUC-60 were negatively correlated. There were also negative correlations between V_e and VEGF-A, Ve and Ang-2, V_e and Ang-1, and V_e and HGF at this time point.

A novel finding from this thesis was the development of a statistical method which successfully combined DCE-MRI and DWI biomarkers to assess heterogeneity in liver metastases.

By developing and applying this method to a sub population of our trial patients with multiple liver metastases, we identified significant differences within subjects bearing multiple liver metastases, mean value 1.39 ($p<10^{-6}$, Chi-squared test) and between subjects bearing multiple liver metastases, mean value 2.23 ($p<10^{-6}$, Chi-squared test). These results were highly significant and indicated greater variability between metastases in different patients, than between multiple metastases in an individual patient.

The prognostic importance of performance status and serum LDH are well established in colorectal cancer. However, the significance of CEA is less clear. A prognostic score developed by Chibaudel, for metastatic colorectal cancer patients receiving first line chemotherapy (Chibaudel, Bonnetain et al. 2011), identified both LDH and performance status as the most important clinical prognostic factors. The same researchers categorised CEA according to normal or elevated values and found this dichotomisation did not reach statistical significance in relation to outcome. In Kohne's analysis of prognostic factors in metastatic colorectal cancer, CEA was categorised into four bands (30, 100, 500, 1000) (Kohne, Cunningham et al. 2002). However this categorisation was not significant on multivariate analyses and the survival difference between the top and bottom group was only 1.6 months. The CEA data for our data was highly skewed towards very high values for some patients, and only 9 patients had values on or below the normal range. Categorising the data around the median value in our population removed some of these outliers, although the median value for our population may not be representative of a typical population, given the high disease burden.

Elevated white cell count, low haemoglobin level, elevated alkaline phosphatase level, presence of peritoneal carcinomatosis and the location of primary tumour are also known prognostic factors in colorectal cancer (Kohne, Cunningham et al. 2002). These data were not prospectively collected as part of this trial which could be regarded as a potential limitation of this study.

An association between certain DCE-MRI parameters and radiological response was observed in the absence of any significant association between the same parameters and progression free survival. The relationship between response rates and survival in patients with metastatic colorectal cancer has been explored in two large meta-analyses (Buyse, Thirion et al. 2000; Johnson, Ringland et al. 2006). Although response rates correlated with overall survival and progression free survival, the correlation was less than would be expected and did not account for the survival differences observed among many of the individual trials examined.

Johnson and colleagues calculated that if a new drug were tested in a trial which included 250 patients with metastatic colorectal cancer an increase in the response rate by 38% would be required for this to translate to a survival benefit. The conclusions drawn from both meta-analyses, were that very large differences in response rates between treatments were needed before this translated to even modest incremental improvements in PFS or OS in metastatic colorectal cancer. This may partly explain why DCE-MRI parameters were more sensitive for radiological response assessment compared to progression free survival.

Using a univariate analysis for progression free survival, VEGF-A was more statistically significant as a continuous variable than as a categorical variable.

This is because the categorisation of variables according to a cut off, typically taken as the median value, although attractive in its simplicity, potentially results in the loss of significant amounts of statistical information regarding the variability in a distribution (Altman and Royston 2006). However, the categorisation of continuous data can be useful, particularly if data are highly skewed, as it will reduce the impact of most outlier values and in this way reduce the risk of false positive results or type 1 error (Altman, McShane et al. 2012).

Our findings that pre-treatment levels of VEGF-A were significantly associated with progression free survival are consistent with larger trials in metastatic colorectal cancer that have recently been reported (Hegde, Jubb et al. 2012; Jurgensmeier, Schmoll et al. 2013). This study adds to the accumulating evidence that VEGF-A is a prognostic factor in metastatic colorectal cancer. We also observed that median values for VEGF-A were significantly higher in our study, 190pg/mL, compared with 98pg/mL, in a recent report from the Horizon investigators (Jurgensmeier, Schmoll et al. 2013). It seems likely that this again reflects the high disease burden in our patient population, although surprisingly, there was no association between VEGF-A and WTV. However, given that WTV is a not a true estimation of disease volume in our population, as it will only encompass disease in the field of view of the MR scan, this latter finding cannot be regarded as conclusive.

Levels of sVEGFR-2 prior to treatment were prognostic for PFS in our patient population, with low levels favouring a longer PFS. Other studies investigating patients with metastatic colorectal cancer treated with cediranib (Jurgensmeier, Schmoll et al. 2013) or bevacizumab (Kopetz, Hoff et al. 2010) have failed to identify any prognostic or predictive significance for sVEGFR-2.

Paradoxically, researchers reporting on a recent trial in breast cancer patients identified that high levels of both VEGF-A and VEGFR-2 were predictive of treatment benefit for bevacizumab (Miles, de Haas et al. 2013). Significantly, this effect was only apparent for patients who received a higher dose of bevacizumab (15mg/kg v 7.5mg/kg), which could explain the contradictory findings in relation to colorectal cancer.

Thus in breast cancer treatment with bevacizumab at 15mg/kg, could abrogate the effect of an otherwise adverse angiocytokine profile. A small phase II study investigating cediranib in hepatocellular cancer also identified VEGFR-2 as a prognostic factor, and found that high levels pre-treatment were associated with a shorter PFS (Zhu, Ancukiewicz et al. 2013). This would be consistent with VEGFR-2 as both a prognostic factor and a potential predictive factor for treatment with bevacizumab. However the findings from our study can only support a prognostic role for VEGFR-2.

A negative correlation was observed between V_p and VEGFR-2 consistent with earlier work from our group which also identified a negative correlation between V_p *and* VEGFR-2 in patients with ovarian cancer following chemotherapy (Mitchell, O'Connor et al. 2010). These results are biologically plausible in that a tumour with a low *Vp* (which is representative of plasma volume) might be expected to be relatively more hypoxic; which would result in HIF-1 mediated up-regulation of hypoxia responsive genes such as VEGF-A and VEGFR-2. It is intriguing that this correlation persisted following treatment with bevacizumab, although it is possible that this was due to a reduction in both V_p and VEGFR-2. At 14 days, there was also evidence of a weak negative correlation between VEGF-A and V_p. It is striking that significantly more correlations between imaging parameters and angiocytokines were evident following treatment with bevacizumab.

Fourteen days after treatment with bevacizumab, Ang-1 and IAUC-60 were negatively correlated. There were also negative correlations between Ve and VEGF-A, Ve and Ang-2, Ve and Ang-1, and Ve and HGF at this time point.

An a priori hypothesis which under pinned this research was that the DCE-MRI derived biomarker K^{trans} would help to stratify patients who were more likely to benefit from treatment with bevacizumab. K^{trans} dichotomised around the median was significant on multivariate analysis for PFS amongst other pre-treatment prognostic factors. In renal cancer, researchers published the largest series of 44 patients who had been investigated using DCE-MRI during anti-angiogenic therapy. They identified that a high baseline K^{trans} and high V_p dichotomised around the median value, both predicted a longer progression free survival in patients before treatment with sorafenib (Hahn, Yang et al. 2008). Interestingly, the median value of K^{trans} of $0.182min^{-1}$ was significantly higher than the median value of K^{trans} in the current study ($0.159min^{-1}$). This could be explained by the fact that renal cancer tumours are highly angiogenic and it seems probable that values of K^{trans} are likely to differ depending on the underlying tumour type under investigation.

One significant limitation of the work presented in this thesis is the choice of analysis method used to deal with multiple tumours in an individual patient. This requires consideration as multiple tumours from an individual cannot be treated as independent variables. Obtaining an average value for each parameter by incorporating all of the tumours analysed for each patient is an accepted method of analysing DCE-MRI data (O'Connor, Jackson et al. 2012). However, averaged values result in over representation of extreme values and are also less representative of a skewed distribution.

Data from this study shows that the median value of K^{trans} from a single baseline visit using individual tumours was 0.142 min^{-1}, significantly less than the median of the average values, 0.158 min^{-1}. An alternative method which could be used to analyse these data without recourse to collating results for each patient would be mixed effects modelling. Although undoubtedly a more complex analysis method, this has the advantage of preserving more information regarding parameter heterogeneity within and between lesions and is likely to more accurately represent the underlying tumour biology.

The assessment of tumour heterogeneity has become increasingly relevant with the development of targeted therapies. Few studies have described the heterogeneity between different metastases in a single organ within an individual, although a high concordance between mutations in liver metastases within the same patient has been reported (Jones, Chen et al. 2008). In contrast, results from a study by Goasguen and colleagues (Goasguen, de Chaisemartin et al. 2009) suggested that not only does significant heterogeneity exist between metastatic deposits in different patients but that there is frequently considerable variation in gene expression and therapeutic response between individual metastatic deposits in the same patient. A recent study by van Kessel et al. (van Kessel, Samim et al. 2013) presented a retrospective analysis of patients with metastatic colorectal liver disease and showed evidence of heterogeneity of radiological response in 35% of cases, associated with a significant reduction in median survival. Other recent work has also identified the complex heterogeneity that arises within tumours, allowing them to adapt to their constantly changing microenvironment. This has been comprehensively reviewed elsewhere in the literature (Marusyk and Polyak 2010; Fisher, Pusztai et al. 2013).

The growing evidence that significant biological variation exists within and between metastatic deposits implies that heterogeneity of tumour response to different therapies might be predicted if appropriate biomarkers can be developed. Repeated or multiple tissue biopsies are clearly impractical giving rise to an increasing need for alternative non-invasive approaches. Imaging biomarkers provide a potential solution offering unique advantages over soluble or tissue-based biomarkers.

Ideally, imaging biomarkers could be used to identify biological or genetic variations to support enrichment of clinical trial data and provide predictive information to guide therapy. However there remain substantive technical problems associated with the use of imaging biomarkers in this context. Identification of biological variability within tumours requires the calculation of reliable and robust imaging biomarkers from each voxel in the tumour. In practice such pixel-by-pixel mapping is associated with significant errors related to physiological movement and measurement bias induced by the biological variation within the tissue.

For example, the accuracy of measurements of blood volume varies systematically with the measured value (Li, Zhu et al. 2000) and the error models associated with many imaging biomarkers demonstrate similar but more complex bias (Li and Jackson 2003). Another significant problem is that the biological variations described are subtle and the effects on individual imaging biomarkers unpredictable. In this study we have examined these problems using repeated baseline scans to identify and correct for non-biological variation in imaging biomarkers. Furthermore we have constructed a statistical framework that allows combination of these corrected variables to increase statistical power. The results demonstrate that: conventional DCE-MRI and DWI metrics have sufficient information content to support identification of biological variation in metastatic deposits within and between patients.

Furthermore, we have demonstrated novel methods to support identification and reduction of non-biological variance in measurements and to support the development of imaging biomarker combinations from different imaging techniques.

Whilst this work supports further studies of tumour heterogeneity it highlights the requirement for technical developments to improve the accuracy of individual imaging biomarkers. Conventional DCE-MRI and DWI studies commonly acquire data during free breathing and, analyse imaging biomarkers on the basis of ROI-derived means. This is clearly inadequate as intra-tumoural heterogeneity is an important feature of tumour biology. The development of improved motion correction techniques and improved understanding of the error models that underlie individual imaging biomarkers will be essential if these techniques are to develop to become useable in clinical trials and personalised therapy. Of particular importance is the development of multi-spectral "tissue signatures" which can be related to individual genetic or biological signatures. This requires not only improvements in acquisition and modeling techniques but also the acquisition of large data sets to provide adequate statistical power for training of the subsequent mathematical models.

The need for a minimum volume of disease in order to ensure sufficient reproducibility of imaging biomarkers is clearly a limitation in the application of imaging biomarkers to clinical trials and currently restricts the use to a subset of the trial population with large volume metastatic disease. However these patients can be challenging to recruit to imaging studies as they are more likely to have disease related symptoms and have a poorer performance status which makes it harder for them to comply with imaging protocols. Furthermore, there is a significant risk that the powerful effect of disease volume as a prognostic factor will dominate any interpretation of additional biomarker data.

The very nature of DCE-MRI or PET imaging studies investigating putative biomarkers demands that patients have a minimum specified disease volume. This should be addressed in future imaging studies so that stratification can be used to help to abrogate for this effect.

A number of areas for future work have been identified as a result of this research. Only a selection of the circulating biomarkers investigated within this study, were reported in this thesis. The next stage of this project would be to complete the analysis of the remaining circulating biomarkers which include: FGFb, PDGFbb, VEGF-C, IL-6, IL-8, KGF, PlGF, VEGFR-1, VCAM-1 and SDF1b. Results would then be investigated in relation to clinical outcome and correlated with DCE-MRI and DWI biomarkers. Future work would also investigate the dynamic changes in imaging biomarkers and angiocytokines following the addition of chemotherapy to bevacizumab. Since circulating angiocytokines were collected every 6 weeks until disease progression, another planned area of investigation would be the longitudinal assessment of these biomarkers which could indicate early progression and resistance mechanisms during treatment with bevacizumab.

Some limitations in relation to the data analysis for this study have already been acknowledged. Further work should identify sub groups of patients with changes in biomarkers after treatment, beyond the co-efficient of reproducibility, calculated using the Bland-Altman method. This would allow investigation of treatment effects at the individual level rather than the group level, which offers more possibility in terms of stratification for treatment. An alternative method of analysing the imaging data presented in this thesis would be the use of the statistical methods developed for the heterogeneity sub-study.

Combining imaging biomarkers and taking account of multiple lesions in an individual would greatly increase the statistical power to detect therapy related changes

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