Novel radioimmunotherapy for lymphoma and solid tumours both as a single agent and in combination with the Vascular Disrupting Agents

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Declaration

I, Gairin Sara Dancey, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Background

Single-agent radioimmunotherapy (RIT) has demonstrated efficacy in B-cell lymphomas but has been relatively disappointing in solid tumours. To improve its efficacy combinations of RIT with new agents are being investigated. One rational combination, from preclinical studies, is RIT and a vascular disrupting agent (VDA).

Aim

To complete two Phase I clinical studies: 1) using single-agent RIT in Hodgkin lymphoma (HL) and T-cell Lymphomas and 2) combining RIT with Combretastatin-A4-Phosphate (CA4P) for solid tumours. Angiogenic cytokines and circulating cells were investigated as potential biomarkers for VDA induced hypoxia.

Methods

Two phase I, open-label, non-randomised dose-escalation clinical trials were completed. In the lymphomas a murine CD25-antibody conjugated to ^{131}I was used in 14 patients. In CEA-expressing gastrointestinal tumours an anti-CEA (carcino-embryonic antigen) antibody, A5B7 was used with CA4P in 12 patients. ELISA (enzyme-linked immunosorbent assay) measured angiogenic cytokines in serum and flow cytometry assessed Tie-2 monocytes and EPC's. Hepatic artery embolisation was used as a model for acute tumour hypoxia.

Results

The ^{131}I-CHT25 study demonstrated efficacy with a response rate of 67% at or above the MTD (maximum tolerated dose). In solid tumours ^{131}I-A5B7 and CA4P produced one minor response with a corresponding tumour marker fall. Erythropoietin, VEGF (vascular endothelial growth factor) and Tie-2 monocytes increased post embolisation and would merit further investigation as potential

biomarkers. Angiopoietin 2 appeared elevated in both malignancy and liver disease and was an independent prognostic factor but did not rise post-embolisation. This supported previous work suggesting angiopoietin-2 was derived from surrounding liver rather than tumour.

Conclusion

Single agent RIT appears effective in lymphoma but further research is required in solid tumours. More potent VDAs have since entered clinical trials but the development of biomarkers to determine response will be vital. VEGF, erythropoietin, Tie-2 monocytes and EPC's would merit further investigation for that role.

Dedication

This work is dedicated to my parents, Richard and Sheila Dancey and my sister Madeleine Dancey.

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Abbreviations

5-FU	5-Fluorouracil
5-HIAA	5-hydroxyindoleacetic acid
AASLD	American Association for the study of Liver diseases
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
AFP	Alpha fetoprotein
AITL	Angioimmunoblastic lymphoma
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APC	Allophycocyanin
APTT	Activated partial thromboplastin time
ASCT	Autologous stem cell transplant
AST	Aspartate aminotransferase
ATLL	Adult T-cell leukaemia/lymphoma
AUC	Area under the curve
bFGF	Basic Fibroblast growth factor
CA19-9	Carbohydrate antigen 19-9
CA4P	Combretastatin-4-Phosphate
CAC	Circulating angiogenic cells
CCL2	Chemokine ligand 2 (monocyte chemoattractant protein 1)
CCR2	Chemokine receptor 2
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDR	Complementarity Determining Region
CEA	Carcinoembryonic antigen

CEC	Circulating endothelial cell
CFU-EC	Colony forming unit – endothelial cells
CFU	Colony forming unit
CH	Constant Heavy
CIRB	Central Institutional Review Board
CL	Clearance
CL	Constant Light
CLIP	Cancer of the liver Italian program
CR	Complete response
CRP	C-reactive protein
CT	Computerised tomography
CTC	Common toxicity criteria
CTCAE	Common toxicity criteria adverse events
CXCR-4	Chemokine receptor 4
CXR	Chest X-ray
DCE-MRI	Dynamic contrast enhanced magnetic resonance imaging
DLT	Dose-limiting toxicity
DMXAA	5,6-dimethylxanthenone-4-aceticacid
DNA	Deoxyribonucleic acid
d-s	Double strand
EANM	European Association of Nuclear Medicine
EBRT	External beam radiotherapy
EBV	Epstein Barr virus
EC	Endothelial cell
ECFC	Endothelial colony forming cell
ECG	Electrocardiography
ECHO	Echocardiography
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial Progenitor Cell
EPO	Erythropoietin
EpoR	Erythropoietin receptor
FAB	Fragment Antigen-binding region
FACS	Fluorescence activated cell sorting
FC	Fragment Crystallisable
FcRn	Neonatal Fc Receptor
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
FGF	Fibroblast Growth factor
FGFBP	Fibroblast Growth factor binding protein
FGFR	Fibroblast Growth factor receptor
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FL	Follicular lymphoma
Flt-1	Fms-related tyrosine kinase-1
Flt3-L	Fms-like tyrosine kinase ligand
FSC	Forward scatter
fT4	Free Thyroxine
FV	Fragment Variable
G-CSF	Granulocyte colony stimulating factor
Gd-DTPA	Gadolinium diethylenetriamine penta-acetic acid
GEP	Gastroenteropancreatic
GGT	Gamma glutamyl transferase
GM-CSF	Granulocyte macrophage colony stimulating factor
Gy	Gray
HA	Hepatic artery
HACA	Human anti-chimeric antibodies

HAMA	Human anti-mouse antibodies
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular cancer
HIF1-α	Hypoxia inducible factor alpha
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HTLV-1	Human T-cell Lymphotropic virus 1
IAUGC	Initial time under Gd-DTPA Contrast agent time curve
ID	Injected dose
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor
INR	International normalised ratio
LET	Linear energy transfer
KDa	Kilo Dalton
KDR	Kinase insert domain receptor
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
MDS	Myelodysplastic syndrome
MDSC	Myeloid-derived suppressor cells
MIRD	Medical Internal Radiation Dose
MMP-2	Matrix Metallopeptidase-2
MMP-9	Matrix Metallopeptidase-9
MR	Minor response / mixed response
MRI	Magnetic resonance imaging

mRNA	Messenger ribonucleic acid
MTD	Maximum tolerated dose
MUGA	Multi-gated acquisition scan
MVD	Microvessel density
NADPH	Nicotinamide adenine dinucleotide phosphate
NASH	Non-alcoholic steatohepatitis
NCEPOD	National Confidential Enquiry into Patient Outcomes and Death
NHL	Non-Hodgkin's lymphoma
NK	Natural killer cell
NLS	Nuclear Localisation Sequence
NSCLC	Non-small cell lung cancer
OBD	Optimal biological dose
Olinda/XEM	Organ level internal dose assessment / exponential modelling
OSEM	Ordered subsets expectation maximisation
PB	Peripheral blood
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered solution
PBSCT	Peripheral blood stem cell transplant
PD	Progressive disease
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll protein
PET	Positron emission tomography
PFS	Progression free survival
PIGF	Placental growth factor
PK	Pharmacokinetics
PR	Partial Response
PT	Prothrombin time
PTCLu	Peripheral T-cell lymphoma unspecified
RBC	Red blood cell

RECIST	Response Evaluation in Solid tumours
RFA	Radiofrequency ablation
rHuEPO	recombinant human erythropoietin
RIC	Radioimmunoconjugate
RIT	Radioimmunotherapy
ROC	Receiver Operating Characteristic
ROI	Region of Interest
RT-PCR	Reverse transcription polymerase chain reaction
scFV	single –chain FV
SD	Stable disease
SDF-1	Stromal cell-derived factor 1
SDR	Specificity determining residue
sIL-2R	Soluble Interleukin-2 receptor
SIP	small immunoprotein
SPECT	Single photon emission computed tomography
s-s	Single strand
SSC	Side scatter
SUV	Standardised uptake value
TACE	Trans-arterial chemoembolisation
TAE	Trans-arterial embolisation
TAM	Tumour associated macrophage
TEM	Tie-2 positive monocyte
TCR	T-cell Receptor
TGF	Transforming growth factor
Tie-2 factor	Tyrosine kinase with immunoglobulin and epidermal growth homology domains-2
TKI	Tyrosine Kinase inhibitor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tregs	Regulatory T-cells

TSH	Thyroid stimulating hormone
TTP	Time to progression
ULN	Upper limit of normal
VDA	Vascular disrupting agent
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VH	Variable heavy
VL	Variable light
VPF	Vascular permeability factor
Vss	Steady state volume of distribution
vWF	Von Willebrand factor
WB	Western blot
WHO	World Health Organisation

Publications

A Phase I Clinical Trial of CHT-25, a ^{131}I-Labeled Chimeric Anti-CD25 Antibody Showing Efficacy in Patients with Refractory Lymphoma. Dancey G, Violet J, Malaroda A, Green AJ, Sharma SK, Francis R, Othman S, Parker S, Buscombe J, Griffin N, Chan PS, Malhotra A, Woodward N, Ramsay A, Ross P, Lister TA, Amlot P, Begent R, McNamara C. Clin Cancer Res. 2009 Dec 15;15(24):7701-7710.

A phase I trial of radioimmunotherapy with 131I-A5B7 anti-CEA antibody in combination with combretastatin-A4-phosphate in advanced gastrointestinal carcinomas. Meyer T, Gaya AM, Dancey G, Stratford MR, Othman S, Sharma SK, Wellsted D, Taylor NJ, Stirling JJ, Poupard L, Folkes LK, Chan PS, Pedley RB, Chester KA, Owen K, Violet JA, Malaroda A, Green AJ, Buscombe J, Padhani AR, Rustin GJ, Begent RH. Clin Cancer Res. 2009 Jul 1;15(13):4484-92. Epub 2009 Jun 23.

Circulating angiopoietin-2 is elevated in patients with neuroendocrine tumours and correlates with disease burden and prognosis. Srirajaskanthan R, Dancey G, Hackshaw A, Luong T, Caplin ME, Meyer T. Endocr Relat Cancer. 2009 Sep;16(3):967-76. Epub 2009 Jun 5.

Imaging in targeted delivery of therapy to cancer. Dancey G, Begent RH, Meyer T. Target Oncol. 2009 Sep; 4(3): 201-217. Epub 2009 Oct 8

Serum angiopoietin-1 and 2 as diagnostic and prognostic markers in cirrhosis and hepatocellular cancer. Dancey G, Roughton M, Jacques J, O'Bierne J, Patch D, Burroughs AK, Meyer T. Submitted to J. of Hepatology

Presentations

Serum Angiopoietin-2 is an independent prognostic risk factor for patients with hepatocellular cancer. **Gairin Dancey,** Michael Roughton, Lisa Jacques, James O'Bierne, AK Burroughs, Tim Meyer. Poster presented at International Liver Cancer Association meeting, Milan, September 2009.

Changes in Angiopoietin-1 and -2 and Tie-2 positive monocytes in patients with hepatocellular cancer undergoing transarterial embolisation. **Gairin Dancey,** Mark Lowdell, Richard Begent, Andrew Burroughs, Tim Meyer. Poster presented at ESH Stroma consortium International conference on Vascular targeted therapies in oncology, Mandelieu, October 2007.

Efficacy in a Phase I trial of radioimmunotherapy with ^{131}I chimeric antibody (CHT25) to the IL2 receptor in refractory lymphoma. **Dancey G,** Violet J, Othman S, Parker S, Green AJ, Sharma SK, Francis RJ, Buscombe J, Griffin N, Chan P, Amlot P, Malhotra A, Woodward N, Lister TA, Begent RH, McNamara C. Poster presented at NCRN meeting September 2007.

Phase I trial of a ^{131}I anti-CD25 chimeric antibody for patients with relapsed or refractory lymphoma. **Dancey G,** Violet J, Othman S, Parker S, Green A, Sharma S, Francis R, Griffin N, Chan P, Amlot P, Malhotra A, Woodward N, Lister TA, Isaacson P, Ramsay A, Buscombe J, Begent RH, McNamara C. Oral Presentation at ASH meeting Atlanta 2009.

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Chapter One Introduction to radioimmunotherapy

Cancer remains the second commonest cause of death in the United Kingdom; only circulatory disease is more prevalent. Cure may be possible in localised disease with a combination of surgery, radiotherapy and chemotherapy (1) but is not commonly possible in disseminated disease (2), with the exception of germ cell tumours (3), haematological malignancy (4) and more rarely ovarian epithelial tumours. High-dose chemotherapeutics have not improved the cure rate in common solid tumours (5) either because a tumouricidal dose cannot be delivered due to toxicity or because tumours become drug-resistant.

Chemotherapeutics are not specific to cancer cells, instead targeting generic processes such as cell division. All rapidly dividing cells are therefore affected and toxicity can be severe. The 2008 National Confidential Enquiry into Patient Outcomes and Death (NCEPOD) considered patients dying within 30 days of chemotherapy and concluded treatment had hastened or caused death in 27% (6). Recent research has focused on the need for more targeted agents such as radioimmunotherapy (RIT).

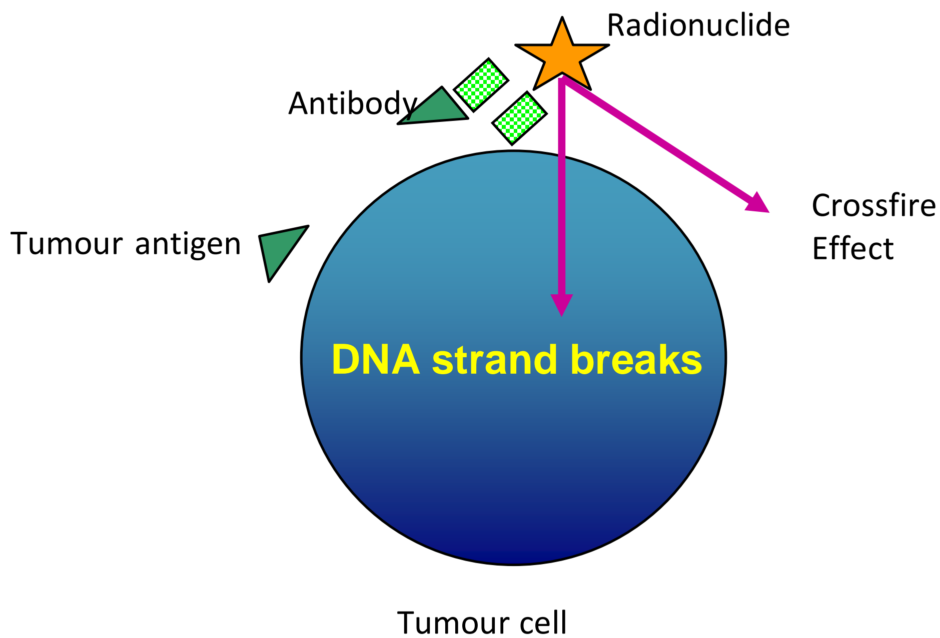
1.1 Targeted agents

Monoclonal antibodies contain a targeting moiety and an effector region capable of causing tumour cell death. Cytotoxicity can be augmented by conjugation to an additional effector molecule like a radionuclide, immunotoxin, chemotherapeutic or cytokine. In this chapter, due to considerations of space, only radioimmunotherapy will be considered further.

1.2 Radioimmunotherapy

Radioimmunotherapy (RIT) is the combination of a monoclonal antibody and a radionuclide. The combination of a radioisotope and antibody is a radioimmunoconjugate (RIC). In addition to targeting cells with the appropriate antigen nearby antigen-negative cells are affected via the 'cross-fire' and 'bystander' effects.

Figure 1.1 Radioimmunotherapy



The 'cross-fire' effect occurs as radiation may be deposited away from the target cell (7). Radiation-damaged cells also cause 'bystander' effects on neighbouring cells via signalling mechanisms which may produce cell death (8). These effects may be advantageous if they affect relatively inaccessible or antigen-negative malignant cells but if they damage healthy cells toxicity will result. The most sensitive organ to RIT is the bone marrow (9) and in non-myeloablative RIT bone-marrow sensitivity is often dose-limiting.

Additional advantages of RIT include the potential presence of a gamma-emission allowing distribution and targeting to be assessed via imaging. Cell internalization of the antibody-antigen complex may also not be required.

1.2.1 Choosing a radionuclide for RIT

A variety of radionuclides have been considered for incorporation into a RIC (radioimmunoconjugate) but only ^{131}I and ^{90}Y are in current widespread use in RIT. The physical characteristics of some radioisotopes considered for RIT are in Table 1.. Bone-seeking radionuclides such as ^{32}P and ^{90}Sr used in metastatic bone disease are not considered here.

When selecting a radionuclide the emission type is considered as well as the linear energy transfer (LET) and the physical half-life. The RIC needs to be compatible with the tumour localisation rate of the selected antibody and with the character of disease to be treated (minimal residual disease or large-volume metastatic) (10, 11). No ideal radionuclide for RIT currently exists.

Table 1.1 Radionuclides considered for radioimmunotherapy

Radio-nuclide	Emission type	Half-life (days)	Mean Range (mm)	Energy E_{max} (keV)	Imaging possible?	Comment
^{131}I	β^- / γ	8	0.4	0.81	Yes	Inexpensive Radiation safety Dehalogenation
^{90}Y	β^-	2.7	2.76	2.3	No	Difficult imaging Bone seeker
^{177}Lu	β^- / γ	6.7	0.28	0.5	Yes	Bone seeker
^{186}Re	β^- / γ	3.8	0.92	1.1	Yes	
^{188}Re	β^- / γ	0.7	2.43	2.1	Yes	
^{67}Cu	β^- / γ	2.6	0.6	0.57	Yes	Long tumour retention Problematic chelator
^{212}Bi	$\alpha / \beta^- / \gamma$	0.04	0.04-0.1	6.09	Yes	Effective in hypoxia Unstable daughter product
^{211}At	α	0.3	0.04-0.1	5.87	Yes	Effective in hypoxia Unstable daughter product
^{225}Ac	α / β	10	0.04-0.1	5.83	Yes	

1.2.2 Beta-emitters

Radionuclides in current use predominantly emit β-particles. These have a long and variable range but a relatively low LET across that range. An extensive ‘cross-fire’ effect is seen (10, 11). Beta-emitters with a particularly long range, such as ^{90}Y, are theoretically more suited to bulky tumours or heterogeneous antibody expression. In minimal disease, most of the energy from long range emitters may be deposited outside the tumour (12, 13). A mathematical model found the optimum size for curability assuming a homogenous radionuclide distribution was 34 mm for ^{90}Y and 3.4 mm for ^{131}I (13).

Radio-metals such as ^{90}Y or ^{177}Lu become trapped in the cell and are more resistant to break-down (14) but have complex radiochemistry and require chelators such as tiuxetan to bind antibody (15). The halogens such as ^{131}I can be conjugated to antibody relatively easily but ^{131}I is degraded after endocytosis (14) with a natural affinity for the thyroid. Potassium iodide administration during RIT is required to prevent gland failure. The radiometal ^{90}Y preferentially deposits in bone, increasing the risk of myelotoxicity (7).

^{131}I produces high-energy γ-emissions, requiring in-patient shielding post-therapy, adding to cost and restricting therapy to self-caring patients. Pure β-emitters such as ^{90}Y avoid this problem but their distribution cannot be assessed directly via a γ–camera, although ^{111}In can be substituted (16). Both ^{177}Lu and ^{67}Cu have γ-emissions of lower energy than ^{131}I and show potential for use in RIT (11). A study of the three radioisotopes ^{131}I, ^{90}Y and ^{67}Cu found higher tumour doses with the first two but a better normal tissue to tumour ratio with ^{67}Cu with an improved therapeutic window (14).

1.2.3 Alpha-emitters

Alpha-emitters have a short range, of about 1-2 cell diameters (50-60 μm) and a high LET across that range (approximately 400 fold higher than β-emitters) so a cell can potentially be killed by a single interaction (11). These features make them potentially suitable for accessible tumours with homogenous antigen expression such as leukaemias (17), tumour vasculature antigens (18) or minimal residual disease (19). Difficulties in the production, labelling and dosimetry of α-emitters have so far proved challenging either due to their short half-lives (20) or for ^{225}Ac, with its longer half-life there is a problematic decay pattern with daughter compounds generating toxicity.

Clinical trials of α-emitters have been performed. ^{213}Bi conjugated to an anti-CD33 antibody in 18 patients with relapsed myeloid leukaemia demonstrated tumour targeting with a tumour: normal tissue ratio 1000 fold higher than with β-emitters. Circulating blast levels fell in 14 of 15 patients and bone marrow blasts in 14 of 18 (17). In melanoma ^{213}Bi conjugated to anti-MCSP (melanoma-associated chondroitin sulphate proteoglycan), an antigen expressed on both melanoma cells and pericytes, gave a response rate of 14%, attributed to damage of the more easily accessible pericytes rather than cancer cells (18, 21).

1.2.4 Auger emitters

Auger electrons are emitted when a nuclide decays with electron capture triggering emissions of orbital electrons (auger electrons) and gamma rays. They have short ranges of 1-2μm and a high LET, however they need to be internalized into the cell, preferably adjacent to the nucleus, to be most effective (22). If internalized their high LET means they can be profoundly cytotoxic. Their ‘cross-fire’ effect is negligible although a significant ‘bystander’

effect may be present. They are relatively non-toxic to normal body cells that do not bind and internalize the RIC.

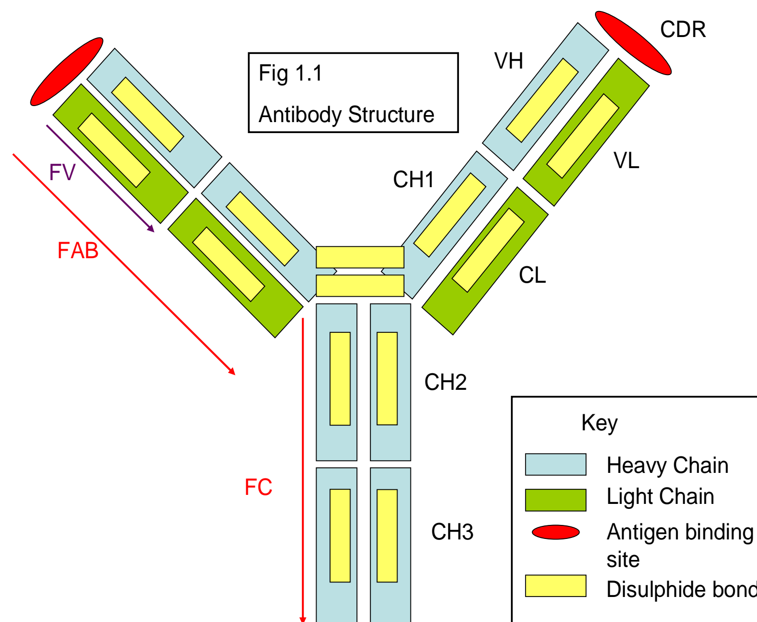
Clinical trials have shown benefit with auger emitters. For example Anthony et al treated 27 patients with gastroenteropancreatic (GEP) neuroendocrine cancers who had failed all conventional therapies, with ^{111}In-pentetreotide. Clinical benefit was seen in 62% with objective responses in 8%, hormonal biomarkers fell in 81% and tumour necrosis occurred in 27% (23). Recent work to improve auger therapy has led to the development of fusion proteins incorporating a nuclear localisation sequence (NLS) to the RIC. The NLS allows for an enhanced translocation of the bulky RIC towards the nucleus where DNA (Deoxyribonucleic acid) damage will be greater. Initial in-vitro and animal work of this technique look promising (24, 25).

1.2.5 Antibodies for radioimmunotherapy

In 1902 Paul Ehrlich used the phrase ‘a magic bullet’ to describe the concept of a specific anti-serum that distinguished healthy from diseased tissue and targeted the latter (26). The concept became a reality with the discovery of the antibody.

Figure 1.2 Antibody structure

Antibody structure of two heavy and two light chains linked by disulphide bonds to form a Y shaped structure. VH = Variable Heavy, VL = Variable Light, CH = Constant Heavy, CL = Constant Light. Fv = Fragment variable, FAB = fragment antigen-binding region, FC = Fragment crystallisable region.



Two sites allow antibody function. The fragment antigen-binding region (FAB) is either side of a flexible hinge, allowing a single antibody to bind two antigens generating cross-linking, aggregation and a faster clearance. The Fc (fragment crystallisable) region activates the host immune system.

Therapeutic antibodies were initially polyclonal until Kohler and Milstein discovered the hybridoma method to isolate monoclonal antibodies (27). Currently eleven unlabeled antibodies have been licensed by the FDA (Food and Drug Administration) as anti-cancer therapies and a further fifteen are licensed for other indications (see Table 1.1).

Table 1.1 Antibodies currently licensed as cancer therapeutics by the FDA

Antigen	Antibody	Trade Name	Type	Tumour targeting
CD20	Rituximab	Mabthera™	Chimeric	B-cell lymphoma
Her2/neu	Trastuzumab	Herceptin™	Human	Breast
VEGF*	Bevacizumab	Avastin™	Human	General
EGFR**	Cetuximab	Erbitux™	Chimeric	General
CD33†	Gemtuzumab	Mylotarg™	Human	Acute Myeloid Leukaemia
CD52	Alemtuzumab	Campath™	Human	B-cell Chronic lymphocytic leukaemia
EGFR**	Panitumumab	Vectibis™	Human	Metastatic colorectal cancer
CD20	Ibritumomab tiuxetan	Zevalin™ (with ^{90}Y)	Murine	B-cell lymphoma
CD20	Tositumomab	Bexxar™ (with ^{131}I)	Murine	B-cell lymphoma
CD20	Ofatumumab	Arzerra™	Human	Chronic lymphocytic leukaemia
CTLA-4	Ipilimumab	Yervoy™	Human	Melanoma

* VEGF (Vascular Endothelial Growth Factor)

** EGFR (Epidermal Growth Factor Receptor)

† Subsequently withdrawn due to toxicity.

Antibodies induce cell death via activation of the host immune system (antibody dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody dependent cellular phagocytosis (ADCP)) or by disruption of vital pathways for tumour cell survival. Resistance can develop (28), cancer patients may have an impaired immune response (29) and tumour penetration by these bulky molecules may be poor.

I shall now briefly consider the particular radiobiology of RIT and the challenges this may present before considering alternative methods to improve RIT for the clinic.

1.3 The radio-biology of radioimmunotherapy

When ionizing radiation interacts with a cell, DNA damage may occur. Double strand (d-s) DNA breaks are harder to repair as they may lack the intact DNA to act as a template. If repair is unsuccessful, cell-death usually occurs on first mitosis. Radionuclides with a high LET have an increased chance of causing d-s breaks (7, 22).

1.3.1 LET and the dose rate effect

Sub-lethal DNA damage can be repaired. If however a second interaction occurs to that same strand or a separate ionising particle affects the opposite strand before repair is completed, cell death is likely to result. This concept gave rise to the dose rate effect where the higher the frequency of ionising interactions, the fewer repairs can be completed and the greater the probability of lethal damage (7). RIT has a low dose-rate that declines with time compared to external beam radiotherapy (EBRT) and brachytherapy. For example RIT has a dose-rate of < 10-20cGy/hr compared to approximately 50cGy/hr with brachytherapy (30).

1.3.2 Hypoxia and RIT

Hypoxia increases the resistance of tumour cells to radiation, particularly β-emitters. In contrast oxygen leads to peroxide formation which prevents

tumour cell DNA repair and subsequent recovery. In RIT the hypoxia commonly seen in solid tumours contributes to resistance to therapy (31).

1.3.3 Dose heterogeneity

The absorbed tumour dose from RIT is often heterogeneous across a tumour due to considerable variability in antigen expression and antigen inaccessibility. Micro-thermo-luminescent dosimeters in B-cell lymphoma xenografts found the absorbed dose varied by up to 400% (32) allowing possible tumour escape and repopulation.

1.3.4 Dosimetry for Radioimmunotherapy

Radiation dosimetry aims to calculate the absorbed dose in a tissue that is deposited secondary to an administered activity of radiation. For EBRT clear relationships exist between absorbed dose, toxicity and tumour response which allow accurate treatment planning. These relationships are less clearly defined for RIT. It is possible to give a tracer dose of the intended RIC to allow predictive dosimetry to determine the most appropriate administered activity. This individualised therapy model for RIT remains challenging (33, 34). Currently tracer studies set limits on whole body dose to 0.75-3 Gy (bone marrow tolerance) and 20-30 Gy for other organs.

1.3.4.1 Methodology for dosimetry

Serial imaging calculates the radioactivity in tumour and normal tissue after RIC administration (34). If the RIC has a γ-emission, a γ-camera can be used. SPECT (single photon emission computer tomography) imaging can define regions of interest (ROI) and determine radioactivity uptake and clearance in normal tissues or tumour as a function of time (cumulated activity). The volume or

mass of tissue within the ROI, calculated using CT (Computed tomography), together with the cumulated activity give the activity concentration. Serial imaging over time allows the area under the curve (AUC) of the time-activity curve to be calculated giving the total number of disintegrations (number of particles created) during therapy in the ROI and allowing the calculation of total absorbed dose.

The most widely used method uses the MIRD (Medical Internal Radiation Dose) Committee schema (35). This assumes that absorbed doses to patients can be calculated from radioactivity distributions within standard phantoms including organs of standard size, shape, position and a homogenous consistency with radioactivity evenly distributed. Absorbed doses per unit administered activity are calculated as the product of the residence time with an S value. The residence time is calculated by dividing the cumulated activity (i.e. the area under the time-activity curve) in the tissue by the injected activity. The S value includes information on the mean energy of each particle emitted during radioactive decay together with the fraction of energy from source organs (organs containing activity), which is either absorbed internally or transmitted to neighbouring organs. The S values for many radionuclides and source-target organs are available for reference (36).

This method ignores the natural variability of organ size, shape and position (37). For example, spleen size in lymphoma patients is highly variable with recorded measurements from <50 g to >600 g compared to 183 g in MIRD (38). MIRD does not consider the radiation dose to normal organs derived from tumour or the dose to tumour from normal organs, although it includes the dose between normal organs. Longer range γ-emissions that may be deposited further from tumour are not included (39).

The MIRD assumption of a homogenous activity distribution is not consistent with evidence of marked heterogeneity at both the tissue and cellular level (37). The kidney has a non-uniform distribution due to its structure and absorbed doses for cortex and medulla should be estimated separately. Fused images using SPECT-CT and PET(Positron emission tomography)-CT allow more accurate estimations of tumour or organ size, shape and position (39).

1.3.4.2 Dosimetry in clinical practice

Current dosimetry methods give only weak correlations between absorbed dose and toxicity or response. If the relationship was more predictable a tracer dose could determine the therapeutic injected activity (39). Dosimetry is used in the therapy of metastatic thyroid cancer with ^{131}I. A fixed administered activity of 7.4 GBq, exceeded the maximum tolerable activity in 22-38% of patients older than 70 (8-15% in those younger) (40). European Association of Nuclear Medicine (EANM) guidelines remain empirical, recommending administered activities between 3.7-7.4 GBq with adjustments determined by dosimetry. In other solid tumours such as neuroendocrine tumours (41) and neuroblastoma (42) relationships exist between tumour absorbed dose and response but wide variability precludes truly individualised therapy.

In indolent B-cell lymphoma pre-therapy tracer scans are used for ^{131}I-tositumomab but not for ^{90}Y-Ibritumomab Tiuxetan which has a more predictable excretion. De-halogenation of ^{131}I is variable between patients leaving free iodine to concentrate in stomach and thyroid before renal excretion. Excretion varied by 46-90% at 48 hrs between patients. This variability in excretion could be predicted by a tracer dose. The radio-metal ^{90}Y is more stable with a more predictable excretion and administered activity can generally be determined by patient weight (43).

1.3.5 Improving the success of radioimmunotherapy

Considerable interest exists in RIT but challenges remain before it can be considered an effective therapy, particularly in solid tumours. Antibody engineering is helping to improve pharmacokinetics and immunogenicity but other problems remain.

1.3.6 Pharmacokinetics, uptake and clearance

To be effective a RIC needs to diffuse homogenously and in high concentration throughout tumour, but remain at low concentration in normal tissues. A bulky IgG (Immunoglobulin G) antibody diffuses slowly from circulation into tumour exposing normal tissues to greater toxicity. Tumour uptake in a mouse model of an antibody against CEA (Carcinoembyronic antigen) called A5B7, was 10-15% ID/g by 24 hours in subcutaneous lesions and large metastatic lesions although higher in small metastatic lesions (44).

Antibody engineering has produced fragments with faster tumour uptakes, clearance and more homogenous distributions in tumour than whole antibodies (45). The smallest fragments are the FV's, which are unstable. A synthetic linker between heavy and light chains gives an scFV (single chain FV) with a molecular weight of 25 kDa (kilo Dalton) (46). These scFv's have an improved tumour to normal tissue ratio (47, 48) but their rapid clearance gives a low absolute tumour absorbed dose. Their low molecular weight may lead to renal filtration, increasing the risk of renal toxicity. Intermediate size fragments are being investigated such as the minibodies (49) and small immunoproteins (SIP's) (50) which have lower tumour clearance and lack renal filtration.

1.3.7 Antibody affinity and avidity

Antibody affinity expresses the strength of interaction between a single antibody site and its specific antigen epitope, including spatial fit, interaction size and charge. The avidity is the functional binding between the antibody and its antigen. It is the sum strength of all the affinity bonds (functional affinity) and takes into account multiple binding interactions with different epitopes.

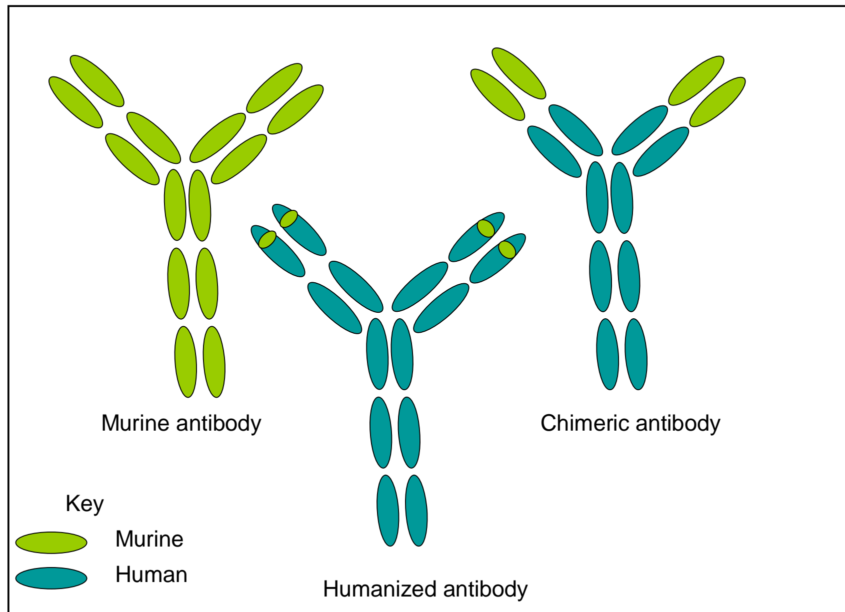
Antibody engineering can increase affinity and extend binding duration (51, 52). At very high affinities antibody remains bound to the nearest antigen and stays trapped in the peri-vascular space rather than diffusing through tumour. This effect is the 'binding-site barrier' (53). It cannot be overcome by increasing administered dose due to the toxicity of the radioisotope.

1.3.8 Immunogenicity

Antibodies were initially murine in origin with less ability to activate the human immune system (54) and a more rapid excretion due to failure to bind the human salvage receptor FcRn (neonatal Fc receptor) (54). At two to three weeks post-exposure human anti-mouse antibodies (HAMA) are formed in 50% of patients with an intact immune system and nearly always with repeat therapy (55). HAMA formation can aid antibody clearance (56) and lead to flu-like symptoms, urticaria or mild bronchospasm on retreatment due to IgG reactions (57).

Immunosuppressive therapies, such as cyclosporine A, can delay but not prevent HAMA formation and have additional toxicities (55). An alternative approach is to replace murine antibody protein with human.

Figure 1.3 Adapting antibodies to reduce immunogenicity



Chimeric antibodies have human constant regions and murine variable regions. As most HAMA form against the Fc region a chimeric antibody significantly reduces immunogenicity (58). However human anti-chimeric antibodies (HACA) may form against the remaining murine protein including the antigen-binding site (anti-idiotype antibodies). Chimeric antibodies have a longer half-life than murine (up to 10 days), which may not be effected by repeat exposure.

Humanized antibodies retain murine complementary determining regions. Antibodies can develop against their specificity determining residues (SDR) but are often of low titre and do not preclude re-treatment. Humanized and chimeric anti-cancer antibodies in clinical use are listed in Table 1.1

1.3.8.1 Two-step approaches

A two-step approach involves separating the administration of the antibody from the radionuclide. The antibody is allowed time to localize and bind to tumour before a low molecular weight radionuclide is given that binds to that initial antibody (59). This method gives high tumour to normal tissue ratios and allows safer escalation of the injected activity (59, 60). Rapid radionuclide excretion gives a potential for renal toxicity and the administration of two or more compounds can increase immunogenicity. The different systems developed are more fully discussed in a review by Goldenberg et al (60).

1.3.8.2 Selecting the right tumour

RIT appears most effective in highly vascular tumours with readily accessible and homogeneously expressed antigen (61). These include haematological malignancy and adjuvant therapy for minimal residual disease.

Solid tumours have a poorly organised vasculature with no accompanying lymphatic vessels (62, 63) and an elevated mean interstitial pressure (64) that obstructs antibody diffusion. Tumour cells are often poorly perfused and acidotic, hypoxic and radio-resistant (65). Antigen may be heterogeneously expressed and tumour tissue inherently more radio-resistant. All these challenges make RIT more difficult in solid tumours.

1.3.8.3 Selecting the right antigen to target

The identification of a suitable target antigen is vital for the ultimate success of antibody therapy (66). An ideal antigen is highly and homogeneously expressed on cancer cells although the number and density of antigens required varies.

Only 100,000 copies of the CD20 (Cluster of Differentiation) antigen are required per cell in B-cell lymphomas (67) but 2.3 x 10^6 copies per cell are needed of the Her2/neu receptor in breast cancer (67).

The antigen should be relatively tumour cell specific. B-cell lymphomas possess a unique immunoglobulin idiotype that proliferates as a single unique clone (68). In most cases tumour antigen is not restricted to tumour cells but is found at a lower density of expression on healthy cells, is expressed during a particular developmental stage, is relatively inaccessible (69) or found on cells that are continuously replenished and therefore non-essential (70).

An ideal antigen is not shed or secreted, which may enhance renal clearance due to complex formation in the circulation (71). The administration of the RIC cannot be increased to overcome this antigen 'sink' although unlabeled antibody can be administered prior to the RIC. Shedding or secretion is less problematic for unlabeled antibodies and the shedding of Her2/neu has not impaired targeting by trastuzumab (67). In addition the ideal antigen should be expressed on the majority of tumours of a particular cancer type or a diagnostic test be available allowing patient selection (72). It should not be lost with tumour de-differentiation and may be ubiquitous across many tumour types (67).

1.3.8.4 Selecting the right patient

Typical patients in Phase I clinical trials have resistant bulky tumours. These patients are unlikely to be ideal candidates for RIT which is likely to be most effective in small volume, minimal residual disease (73) or haematological malignancy (74).

1.4 First Hypothesis

Considering the previously published work described here we would expect that single agent RIT would be effective in haematological malignancy. In Chapter Two I shall present the results of our phase I clinical trial of a chimeric anti-CD25 antibody conjugated to ^{131}I in patients with CD25 expressing lymphomas (predominantly Hodgkin's lymphoma (HL) but also T-cell lymphomas).

Lymphomas are highly radio-sensitive tumours; they are highly vascular with less necrosis and hypoxia than solid tumours. Antibody penetration is therefore easier with higher absorbed doses anticipated. Although most of the previous work is in indolent B-cell lymphomas it is likely that Hodgkin's lymphoma shares those characteristics and therefore shares sensitivity to RIT.

Generally both HL and T-cell lymphomas have a higher proliferation rate than indolent B-cell lymphomas. As RIT delivers radiation at a low-dose-rate the cell-kill it induces might be inadequate to induce prolonged responses. If the results are consistent with the previous work a sub-group of patients may have durable responses.

1.5 Radioimmunotherapy in Lymphoma

Much of the current information about RIT for lymphoma is derived from treating patients with indolent B-cell lymphoma. Two radio-labelled antibodies are licensed; ^{90}Yttrium-ibritumomab tiuxetan (^{90}Y-IT) (Trade-name Zevalin®) and ^{131}Iodine-tositumomab (^{131}I-T) (Trade-name Bexxar®). As many characteristics of RIT appear to be class effects, it is worth looking at these 2 agents in greater detail. Then in Chapter Two a brief introduction will consider the CD25 receptor in greater detail before presenting the results of our phase I study targeting CD25-expressing lymphomas with ^{131}I.

1.5.1 Indolent B-cell Non-Hodgkins-lymphoma (NHL)

Indolent B-cell lymphomas have a typically slow disease course with a relapsing, remitting pattern. With each relapse, response rate and duration of response decrease (75). Cure is possible in localized disease (76) but 80% present when it is disseminated. Combination chemotherapy including high-dose therapy with autologous stem cell transplant (ASCT) has had little impact on survival (77) and allogeneic transplant is only suitable for young, fit patients (78). Median survival has remained stable at 8-10 years (75).

1.5.2 Rituximab for indolent B-cell lymphomas

Rituximab is a chimeric antibody to the cell surface receptor CD20 found on both malignant and normal B-lymphocytes but not on progenitor or stem cells. Cytotoxicity is via ADCC and activation of complement with induction of apoptosis in vitro (79). It is active in indolent B-cell lymphoma with a response rate of 48% in relapsed disease (80) rising to 95% when given first-line in combination with chemotherapy (81). Although encouraging, the majority of patients either fail to respond or subsequently relapse.

1.5.3 ^{131}I-tositumomab (^{131}I-T) (Bexxar™)

^{131}I-T is a murine anti-CD20 antibody conjugated to ^{131}I with a variable renal excretion requiring pre-therapy dosimetry. Kaminski et al treated 59 patients with relapsed or refractory disease. The response rate was 71% (83% in low grade or transformed disease), with a median progression free survival of 12 months in 42 responders (82). Re-treatment was feasible and induced responses in 9 of 16 patients. Response in the first line setting was 95% (83).

Toxicity was generally mild and included infusion reactions, nausea and tiredness. HAMA occurred in < 10% of heavily pre-treated patients but 63% of those treated first-line (83). Potassium iodide was given to reduce thyroid uptake of radioactive iodine. With this precaution the incidence of thyroid dysfunction (defined as either an elevated TSH (thyroid stimulating hormone) or requiring thyroid replacement medication) was less than 10% (82, 84). The peripheral blood count nadir was at approximately 6 weeks with grade 4 neutropenia seen in 14%. The incidence of acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS) was reviewed in a meta-analysis and not considered elevated above anticipated levels (85).

1.5.4 ^{90}Yttrium-ibritumomab tiuxetan (^{90}Y-IT) (Zevalin™)

The first RIC to receive a license for relapsed or refractory indolent B-cell lymphoma was ^{90}Y-IT. It comprises a murine antibody to the 2B8 epitope on CD20 (rituximab is the chimeric form). Its predictable excretion allows a standard injected activity without pre-therapy dosimetry (86). A randomized study of ^{90}Y-IT versus single-agent rituximab improved response rates from 56% to 80% (74). Response rates were higher in smaller volume disease and at first relapse but also seen in patients resistant to rituximab (74%) (87).

It was generally well tolerated. Haematological toxicity correlated with the number of lines of prior chemotherapy and the extent of marrow involvement with lymphoma (88). Grade IV neutropenia was seen in 30% of patients receiving the standard dose of 0.4 mCi/kg (14.8 MBq/m^2) but was associated with sepsis in only 3% (88). This low rate was attributed to the lack of mucosal damage and preservation of NK (Natural Killer) and T-cell function (89). AML / MDS rates were equivalent to historical controls for chemotherapy-exposed patients (90). Elderly patients tolerated RIT as well as the young (91); chemotherapy was possible after RIT with no additional toxicity (92).

1.5.5 Lessons for the CHT25 clinical trial

Past experience with ^{90}Y-IT and ^{131}I-T provides useful information for our study. Additional benefit was seen with the RIC when compared to the antibody alone (rituximab). As CD25-expressing lymphomas share biological characteristics with B-cell lymphomas (an inherent radiosensitivity, relatively accessible tumour cells, a good vascular supply and a low level of necrosis and hypoxia), we have hypothesized they will also be sensitive to RIT. Their higher rate of proliferation might however reduce the extent of that response. In the trials of B-cell lymphomas a sub-group of patients had long-term durable responses with the hope of cure in some individuals.

We would anticipate toxicity to be predominantly haematological with bone-marrow suppression dose-limiting. Blood count nadir is likely to occur at 6-7 weeks post infusion. A heavily pre-treated patient group (such as our phase I study) would be more at risk of severe bone marrow suppression. There is likely to be a lower incidence of HAMA or HACA formation however. Reassuringly any subsequent therapeutic options are unlikely to be precluded by RIT.

1.6 Radioimmunotherapy of Solid Tumours

In contrast to the haematological malignancies already discussed, solid tumours appear relatively insensitive to RIT. Multiple trials of single agent RIT in solid tumours have been relatively disappointing (7, 93) with a typical response rate of 10% (93, 94). A number of factors are likely to be contributory:

- A poorer vasculature with absent lymphatics resulting in an elevated interstitial pressure limiting antibody diffusion (64, 95).
- Greater heterogeneity of antigen expression both within and between cells. Areas of under-dosing can lead to tumour re-growth (96).

- Antigen secretion into the circulation with immune complex formation and faster clearance (71).
- Increased hypoxia which is inherently radio-resistant (97)
- Reduced radio-sensitivity of solid tumours (98)
- A preserved immune system capable of mounting a response to murine or chimeric antibody (99).

1.6.1 Colorectal and other CEA-expressing gastrointestinal cancers

To demonstrate the challenges of RIT in solid tumours I shall consider its impact in the targeting of CEA-expressing gastrointestinal malignancy in greater detail.

1.6.1.1 CEA as a target for Radioimmunotherapy

CEA is a useful antigen to target in radioimmunotherapy. It is present on 95% of all colonic adenocarcinomas (100) and a smaller percentage of other epithelial tumours including pancreatic and stomach cancer. In healthy tissue CEA expression is restricted to the apical surface of the microvilli in the colon epithelium, however in malignancy it is expressed on all cell surfaces. A systematically delivered anti-CEA antibody cannot access the luminal surface of the normal colon cells due to the presence of tight junctions, allowing for some selectivity to malignant cells.

In malignancy CEA is secreted into the circulation and can act as a tumour marker. A high serum CEA may reduce tumour targeting as complexes are formed in the circulation and cleared (101).

1.6.1.2 Clinical trials of RIT in CEA-expressing gastrointestinal cancers

RIT has been investigated in both the adjuvant setting and in advanced disease. Studies have tended to be non-randomized with small patient numbers and have used a variety of protocols, radionuclides and antibodies making comparison between them difficult. Most studies are in patients with bulky disease, resistant to conventional therapeutics.

1.6.1.3 Advanced disease

Studies of RIT against CEA-expressing tumours are listed in Table 1.3. All 13 studies were phase I or single arm phase II trials exploring toxicity and efficacy. Median patient number was 16 and only 1 used pre-targeting (102).

Table 1.2 Studies targeting CEA in advanced tumours						
Drug	**Species antibody**	**Fragment or whole**	**No patients**	**Response**	**Comment**	**Reference**
^{131}I-PK4S	Sheep	Polyclonal	16	1 PR	Clearing antibody	Begent et al 1989 (103)
^{131}I-A5B7	Murine	IgG_1/$F(ab')_2$	19	1 CR 1 PR	Repeat administration	Lane et al 1994 (94)
^{131}I-NP-4	Murine	IgG_1	57	1 PR 4 MR 7 SD		Behr et al 1997 (104)
^{131}I-NP-4	Murine	IgG1	13	7 SD 6 PD	Repeat administration	Juweid et al 1996 (105)
^{131}I-COL-1 /^{131}I-CC-49	Murine	IgG_1	14	4 SD	Dual antibody with IFNα-2b	Meredith et al 1996 (106)
^{131}I-IMMU-4	Murine	IgG_1	6	1 PR	With hyperthermia	Mittal et al 1996 (107)
^{131}I-F6	Murine	$F(ab')_2$	10	1 PR 2 SD	With bone marrow rescue	Ychou et al 1998 (108)
^{131}I-FO23C5	Murine	IgG1	10	1CR 2 PR 4 SD		Behr et al 1999 (73)
^{188}Re-MN-14	Murine	IgG_1	11	None	Repeat administration	Juweid et al 1998 (109)
^{90}Y-T84.66	Chimeric	IgG_1	22	2 MR 3 SD	Repeat administration	Wong et al 2000 (110)
^{90}Y-T84.66	Chimeric	IgG_1	21	1 MR 11 SD	With 5-FU chemotherapy	Wong et al 2003 (111)
^{131}I-MN-14	Humanized	IgG_1	21	None		Hajjar et al 2002 (112)
MN-14 x m734 + ^{131}Ihapten	Humanized	IgG_1	35	Not reported	Pre-targeted RIT	Kraeber-Bodere et al 1999 (102)

CR: Complete response, PR: Partial response, MR: Mixed response or minor response

IFN: Interferon, 5-FU: 5 Fluorouracil

Initial studies used murine antibodies. Lane et al (94) compared ^{131}I F(ab')2 fragments with whole antibody. One patient in each arm had a partial response. Juweid et al (105) used fragments of the NP-4 antibody and repeat administration in patients with metastatic disease < 3cm. Most patients became HAMA positive and 7 had stable disease for up to 7 months. The largest trial was by Behr et al (104) of 57 patients of whom 12 were thought to derive clinical benefit and 1 had a partial response. A higher affinity antibody, MN14, conjugated to ^{188}Re in 11 patients generated no responses (109).

Subsequent studies used chimeric or humanized antibodies. A dose-escalation study with the chimeric antibody cT84.6 generated clinical benefit in 5 of 22 patients and saw 2 mixed responses (110). The addition of chemotherapy gave no increased toxicity and a reduction in HACA formation (111). Eleven patients then had stable disease for 3-8 months and 1 had a mixed response. A separate study found dose-escalation with stem cell support was feasible, using the F6 antibody (108). One patient had a partial response in 1 liver lesion and 2 had stable disease.

1.6.1.4 Small volume disease or the adjuvant setting

Mathematical (113) and animal models consider RIT as theoretically more effective in smaller volume disease. Mayer et al used ^{131}I-A5B7 in a mouse model and found smaller tumours had a higher absorbed radiation dose and grew less rapidly (114). Digital autoradiography studies of ^{125}I-A5B7 in mice quantified this difference at 15% ID/g in larger tumours compared to 80% ID/g in smaller (44). High-resolution digital microscopy and fluorescently labelled anti-CEA antibody in nude mice found rapid selective tumour uptake and a strong negative correlation with tumour size at 10 minutes and 1 hour post administration ($p<0.01$) but not at 24 hours (115).

Behr et al considered adjuvant RIT. Nine patients post liver resection for metastatic colorectal cancer were given a single injected activity of ^{131}I-hMN-14. At 36 months, 7 of 9 patients were relapse free compared to historical controls of 33% (116). In addition, 21 patients with chemo-refractory disease < 3cm were treated, 16 had a minor response and 3 a partial response. On relapse, 5 patients were re-treated and 2 had a further response (116).

Liersch et al treated 23 patients who had previously had a liver resection for colorectal cancer with ^{131}I-labetuzumab (a humanized IgG anti-CEA antibody marketed as CEA-CIDE). They were compared to a contemporaneous group of patients in the same institution most of whom received adjuvant chemotherapy (117). Survival was 58 months with RIT compared to 31 in the control group. Ychou et al (118) in a similar patient group gave ^{131}I-F6 F(ab')2 to 13 patients. The disease free survival was 12 months with an overall survival of 50 months and 2 patients remained alive at 127 months.

These studies appear to confirm that RIT does have activity against solid tumours, principally in small volume and minimal residual disease. The treatment of bulky solid tumours however remains challenging. New research is attempting to improve the efficacy of RIT in a variety of ways. Antibody engineering may optimize pharmacokinetics or reduce immunogenicity. New targets may be more effective and accessible for RIT. New combinations are being investigated. These have included combinations with cytotoxic agents, cytokines and radiotherapy.

Our own study is reported in Chapter 3 and utilised the novel combination of a ^{131}I-A5B7 anti-CEA antibody together with a Vascular Disrupting Agent (VDA) in CEA-expressing gastrointestinal tumours. Our study aimed to investigate the hypothesis outlined below.

1.6.3 The Vascular Disrupting agents (VDA's)

Vascular disrupting agents target abnormal tumour vasculature leading to vessel shut down, ischaemia and haemorrhagic necrosis (119). Two groups are currently known. Flavonoids such as DMXAA (5,6-dimethylxanthenone-4-aceticacid) which act via disruption of the actin cytoskeleton and induction of apoptosis. The second group are tubulin-binding agents such as the combretastatins. As our Phase I study used a combretastatin this class of agents shall be considered in more detail in Chapter 3.

1.6.4 Combining RIT with vascular targeting agents

Tumour-associated vessels are abnormal and can be targeted by the class of drugs known as the VDA's. Vessels are poorly organized with absent lymphatics (62) are leaky, tortuous and thin-walled (120) with slow flow that can reverse (121). Nutrient supply is often inadequate and tumour centres become hypoxic and necrotic. Targeting blood vessels is appealing. Shut-down of a single blood vessel can kill thousands of dependent tumour cells, endothelial cells are more accessible than most tumour cells and they may be less heterogeneous (122) with a lower possibility of drug-resistance.

Compounds that destroy tumour-associated vessels already in existence are called vascular disrupting agents (VDA) (119). These agents have profound effects in the tumour centre where blood supply is already marginal. The tumour centre is the compartment that RIT fails to kill due to hypoxic radio-resistance and poor penetration. A theoretical possibility of synergy therefore exists, and has been demonstrated in animal models (123). This evidence led to the design of our Phase I study reported in Chapter 3.

1.6.2 Second hypothesis

From the previous work we hypothesized that RIT against cancer-specific antigens would be ineffective in solid tumours. RIT would be ineffective due to the low absorbed dose achieved due a lack of antibody penetration into tumour, the relative insensitivity to radiation of the malignant cells and the preservation of the immune system allowing immunogenicity to develop.

We hypothesized that the combination of RIT with a VDA that targets tumour-specific blood vessels, would act synergistically in solid tumours leading to an increased efficacy. Synergy was predicted from their differing modes of action, with RIT targeting the outer rim and the VDA the hypoxic poorly perfused centre which is inaccessible to bulky antibodies. Evidence of synergy was previously seen in animal studies where the combination of RIT and a VDA transformed a sub-curative therapy into a curative one (124).

1.7 Biomarkers for the Vascular Disruptive Agents (VDA's)

In chapter 3 we presented the results of a Phase I study of a VDA combined with RIT. One of the challenges faced in this study was the lack of a clear biomarker for the Vascular Disrupting Agents. This lack makes it difficult to efficiently design studies to promote synergy between the two agents. This is especially problematic with combinations involving the VDA's where vascular shut-down may prevent RIT access to the tumour. Currently no easy and reliable method exists to determine the biological effect of these compounds.

Identifying a surrogate biomarker for the VDA's would help determine their optimal biological dose (OBD) where vascular shut-down is maximal. Such a biomarker would be of great use in determining the ideal schedule for drug

combinations. Alternatives such as the maximum tolerated dose (MTD) may be inappropriate for these agents where their maximum biological effect may occur at a lower dose than the MTD. Measuring reduction in tumour size by radiology can underestimate any biological effect in drugs that lead to disease stability rather than shrinkage.

Currently the biomarker used most commonly in clinical trials has been either DCE-MRI (Dynamic Contrast Enhanced MRI) or PET based. There are limitations and challenges with these methods. I shall discuss them further in the next section.

1.7.1 Imaging as a biomarker

Using imaging to assess anti-vascular agents is appealing as it is non-invasive, allows repeat assessment and can directly image the relationship between tumour and blood vessels. Both PET and MRI have been important in the clinical development of VDAs.

1.7.1.2 Magnetic Resonance Imaging

MRI techniques have the advantages of high spatial resolution and a variety of contrast agents with good tissue penetration. Accurate anatomical information can be derived about vessel structure as well as function. The technique uses the rate of diffusion of contrast from micro-vasculature into the tumour interstitial space, which depends on vessel permeability, surface area and blood flow (125). Blood kinetics can then be described using the transfer constant (K^{trans}) and the IAUGC. The concordance between DCE-MRI and alternative methods for assessing vascularity, have been contradictory. Whilst most studies have shown a broad correlation with MVD (Microvessel density) (125) and some with tissue VEGF (Vascular Endothelial Growth Factor) (126), others have not.

DCE-MRI has clinical applications. It can improve cancer diagnosis, monitor treatment and predict response to chemotherapy, radiotherapy, anti-androgens (125) and anti-angiogenic agents (127). Changes on MRI can be detected after 1 therapy cycle and at 2 cycles can determine likely treatment effectiveness.

Disadvantages of DCE-MRI include the lack of a standardized approach to the procedure. In our clinical trial presented in Chapter 3, it was noted that of 88 patients considered for the clinical trial 19 were ineligible as their tumours were not assessable by MRI. It is expensive, time-consuming, technically challenging and currently available in only a small number of centers.

1.7.1.3 Positron emission tomography (PET)

Both PET and SPECT can be used to determine total organ blood flow or organ perfusion (127). An isotope such as ^{15}O is used, which is short-lived, chemically stable and biologically inert. It is given either intravenously as a bolus of ^{15}O-labelled water (128) or by inhalation as ^{15}O labelled carbon dioxide. When the rate of radioactive decay is balanced by infusion the isotope concentration relates to blood flow (127). Tissue concentration (from PET imaging) and arterial concentration (obtained either from direct sampling or from imaging blood in the cardiac ventricles) are both required (127).

Validation for this technique has mostly been from studies of the brain or myocardium rather than malignancy. In addition although blood flow was higher in brain tumour tissue than normal tissue there was considerable variability (129). Small studies have considered blood flow in breast (130), pancreatic (131) cancers and liver metastases (132). High-dose CA4P (Combretastatin-4-Phosphate) caused a significant fall in tumour perfusion by 30 minutes that resolved by 24 hours (133). Alternatively the new VEGF-PET

tracer ^{89}Zr-ranibizumab could delineate the anti-angiogenic effects of sunitinib in a mouse xenograft model (134).

PET is expensive and time-consuming. The short half-lives of the compounds (2 minutes for ^{15}O), require an on-site cyclotron and delivery system. The model assumes that labelled water diffuses freely and homogenously throughout the tumour, which may be inaccurate (135). The value produced is an average with no consideration for fluctuations in flow with time or at the micro-vascular level (136). The model is also less reliable at high flow rates (127).

1.7.1.4 Serum biomarkers

The challenges encountered with the imaging modalities has led to research into alternative biomarkers. One of the most promising areas being investigated are the serum biomarkers. Advantages include the relative ease of collection facilitating serial readings. Compared with imaging the cost is lower and the analysis can be performed centrally allowing its application to multicentre trials.

1.7.1.5 Circulating angiogenic cytokines

Cytokines are soluble and diffusible glycopeptides that can be secreted into the blood, either by tumour cells or associated stromal cells, where they can be measured. They play prominent roles in physiological angiogenesis and are also vital for pathological angiogenesis.

Cytokine release is stimulated by hypoxia. The development of tumour hypoxia (which may be driven by a VDA) leads to accumulation of the transcription factor HIF1-α (Hypoxia inducible factor). HIF1-α regulates the expression of a number of genes involved in angiogenesis as well as those involved in erythropoiesis, glycolysis, iron metabolism and cell survival (137). It is tightly

regulated in normoxic tissues (137, 138). Accumulation of HIF1-α in hypoxia leads to secretion of the angiogenic cytokines. The hypothesis is that these angiogenic cytokines would provide a valid biomarker for the vascular endothelial agents.

To investigate this hypothesis a pilot study was completed of five angiogenic cytokines, VEGF (Vascular Endothelial Growth Factor), Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Erythropoietin (Epo) and basic Fibroblast Growth Factor (bFGF). The characteristics of these angiogenic cytokines and why they were selected are reported with the data from the study in Chapter Four.

In this pilot study, designed to screen for cytokines of potential interest as biomarkers it was decided to use a model for the hypoxia generated by a VDA rather than the VDA itself. The model selected was Trans-arterial Embolisation (TAE) in patients with hepatocellular cancer (HCC).

1.7.1.6 Trans-arterial (chemo) embolisation (TAE)

Trans-arterial embolisation generates extensive tumour necrosis by physically obstructing the relevant branch of the hepatic artery that supplies the tumour (139). TAE is possible due to the dual blood supply to normal liver tissue. Whilst tumour cells mostly derive their blood from branches of the hepatic artery (HA), normal liver cells are supplied mainly by the portal vein.

Selective intra-arterial chemotherapy mixed with lipiodol may be administered prior to embolisation. Chemotherapy agents used include doxorubicin, mitomycin and cisplatin. The combined procedure is called chemo-embolisation (TACE). TAE is known to delay tumour progression and lead to objective responses in between 15-55% of patients (139). Meta-analysis has demonstrated a survival benefit of TACE compared with best supportive care

but not with embolisation alone. It is not clear what the optimum protocol should be or even which is the best chemotherapeutic agent.

1.7.1.7 Why is TAE an appropriate model to use?

Studies have shown that pro-angiogenic cytokines such as VEGF are stimulated post-embolisation secondary to ischaemic necrosis caused by vascular shut-down. Cytokine levels were higher than in the livers of patients who had undergone surgery (140) demonstrating the extent of hypoxia induced. Animal models have demonstrated stimulation of HIF1-α release post embolisation, with secondary release of other angiogenic cytokines such as VEGF (141). This generation of angiogenic cytokines post-embolisation has led to clinical studies investigating the use of the multikinase inhibitor, sorafenib, in the period post-embolisation.

Where embolisation delivers vascular shut-down by physical means, a VDA induces it biologically. The embolisation model has the advantage of a clear and definite time for vascular shut-down. A VDA can have a more unpredictable duration and extent of action on blood vessels. The variability of vascular shut down induced by a VDA is demonstrated in Chapter 3. On a practical level, only small numbers of patients on phase I studies are currently having VDA's making recruiting the required number of patients for a pilot study feasible. As a specialist HCC centre the number of patients having TAE or TACE were a more realistic proposition. Any promising candidates could then be taken forward into multi-centre studies using the VDA's.

1.7.1.8 Hepatocellular Cancer (HCC)

HCC is a highly vascular tumour. It develops secondary to liver cirrhosis at a rate of 3-5% of patients per year (142) although chronic hepatitis B can lead to HCC with no preceding cirrhosis, probably as viral DNA is inserted into the host genome (143).

Cirrhosis itself is a highly vascular condition with up-regulation of VEGF, EGF and TGF-α (transforming growth factor). Chronic up-regulation may lead to hepatocarcinogenesis (144), progressing through dysplastic foci and nodule formation (regenerative then dysplastic) into malignancy. VEGF expression increases causing neo-vascularisation (145). Blood vessels undergo arterialisation with increasing expression of CD34 and CD31 (146) and loss of sinusoidal fenestration. Hypovascular early tumours derive blood from the portal system (139) switching to the hepatic artery as they develop and become more vascular. This dual blood supply allows embolisation to be a feasible procedure without causing catastrophic necrosis of healthy liver tissue.

1.7.2 Hypothesis Three

The third hypothesis is that the known ischaemic necrosis of the tumour secondary to TAE will generate secretion of angiogenic cytokines such as VEGF secondary to activation of HIF1-α. These cytokines will be secreted into the blood where they may have potential as biomarkers to assess the extent of vascular shut down.

TAE causes vascular shut down with secondary ischaemic necrosis and hypoxia via physical means. It is hypothesized that this model can be used to investigate potential biomarkers for the VDA’s which achieve vascular shut down and

hypoxia via biological means. Selected biomarkers that show clear potential can then be assessed using the limited number of patients currently treated with the VDA's.

1.8 Tie-2 monocytes and Endothelial Progenitor Cells as biomarkers

Previous work has shown that a variety of bone-marrow cells are implicated in the promotion of tumour growth and angiogenesis. These include tumour-associated macrophages (TAMs), Tie-2 positive monocytes (TEMs), mast cells, neutrophils, eosinophils, dendritic cells, haemangiocytes, myeloid-derived suppressor cells (MDSC) and vascular leukocytes (147). The two groups I shall consider further here are the Tie-2 expressing monocytes and the Endothelial Progenitor Cells. Both groups are thought to be secreted secondary to hypoxia and may represent a potential biomarker for a VDA.

1.8.1 Monocytes

Monocytes are derived from the haematopoietic pluripotential stem cell. They form 5-10% of blood leukocytes (148) and remain circulating for approximately 1-3 days before moving into tissues.

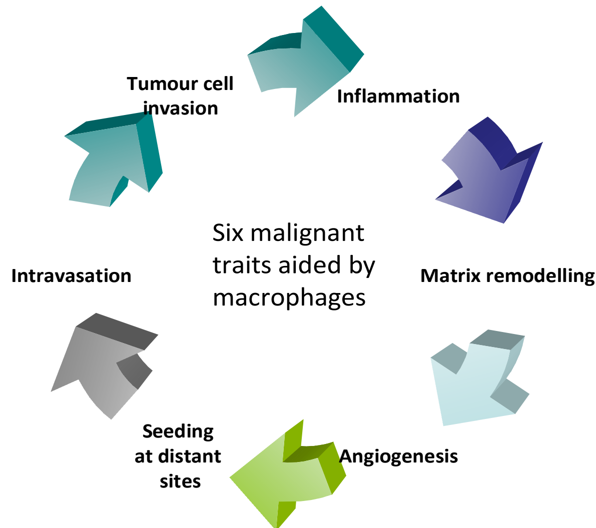
Once in tissues monocytes differentiate into macrophages or dendritic cells depending on the tissue type. Macrophages have a wide range of functions, including phagocytosis, antigen presentation and the release of a wide range of factors including cytokines, chemokines, complement components, coagulation factors, growth factors, enzymes and prostaglandins (149). Circulating monocytes can be attracted to tumours that produce chemokines such as CCL2 (chemokine ligand 2)(formerly monocyte chemoattractant protein 1) and VEGF (150). These are then referred to as tumour-associated macrophages.

1.8.1.1 Tumour-associated macrophages (TAMs)

Macrophages are implicated in the initiation and growth of malignancy. Chronic inflammation, involving macrophages, increases the risk of cancer development and anti-inflammatory agents reduce it (151). Macrophages make up a considerable proportion of tumour mass, and are associated with a poor prognosis in 80% of published studies (152). During tumour development macrophages appear to change character and can enhance metastatic potential. Under the influence of IL-4 and IL-10 they become poor at presenting antigen and instead produced cytokines that suppress T-cell activity (153, 154). This phenotype (termed M2) is normally found in areas of tissue repair and remodelling. The traits aided by macrophages are detailed in Figure 1.4.

Figure 1.4: Malignant traits aided by macrophages

The figure is derived from Condeelis et al (155). The wheel can turn in either direction; macrophages can contribute to each area equally.



Tumour cells become more aggressive after co-culture with TAMs in vitro and approximately 50 genes are up-regulated (156). TAM ablation reduced tumour growth with lower levels of VEGF-A and MMP-9 (Matrix metallopeptidase) (157). In lung cancer TAMs were associated with an increased MVD and a reduced relapse-free survival (156). TAM density is highest in hypoxic tumour where pro-tumour cytokines and mitogenic factors are stimulated (149).

1.8.2 Tie-2 positive monocytes

Approximately 20% of blood monocytes (1-2% of total blood leukocytes) express the Tie-2 receptor and can be referred to as Tie-2 expressing monocytes or TEMs (158). They have been identified in both humans and mice (159) and are strongly pro-angiogenic (160).

1.8.2.1 Identifying a Tie-2 positive monocyte

Typically TEMs are identified by their surface markers. They express the classical monocyte markers of CD14, CD16 and CD11b as well as the haematopoietic marker CD45. They do not express precursor endothelial cell markers such as CD31, CD34, AC133 or CD146 (161). They do not generally express VEGFR2 although Venneri et al did find VEGFR2 expression in a small subset (< 5%) (158). They do not express CCR2 (chemokine receptor 2), the receptor for CCL2 that regulates the recruitment of macrophages to areas of inflammation (158).

They have been found in a variety of tumours including colon, kidney, lung, pancreas and soft tissue sarcomas (158).

1.8.2.2 Attracting Tie-2 Monocytes to tumours

TEMs localise to sites of angiogenesis in mouse models (159) and human tumours. In humans they tend to localize to the peri-vascular space but also to avascular hypoxic tissue (158) and are relatively absent from surrounding non-tumour tissue. This distribution may reflect their attraction to angiopoietin-2 released from endothelial cells (162) secondary to hypoxia (158).

Tumour-associated Tie-2 monocytes have up-regulated Tie-2 expression greater than have circulating Tie-2 monocytes and are more sensitive to Ang-2 (158). Circulating TEMs are thought to be the precursor cells for tumour-infiltrating TEMs. If circulating TEMs are collected and injected into tumour, they appear to share the same pro-angiogenic properties as tumour-infiltrating TEMs (158).

1.8.2.3 Physiological role of Tie-2 Monocytes

TEMs are thought to play a role in tissue growth and regeneration. In mice post partial hepatectomy they are present in granulation tissue and around new vessels (163). In diabetic mice with neuropathy injected TEMs clustered around affected nerves with some reversal of the condition (164).

1.8.2.4 Tie-2 Monocytes in Malignancy

In mouse tumour models the presence of TEMs is pro-angiogenic, leading to more vascularized tumours than in a control group of $CD14^{+}Tie\text{-}2^{-}$ cells (158, 159), which did not affect angiogenesis (158). De Palma et al used a transgenic mouse, Tie-2-tk, devised so all proliferating Tie-2 expressing cells were eliminated on administration of ganciclovir. Mice with TEM ablation showed a reduction in tumour growth and angiogenesis. Tumour infiltration by TAMs was unaffected suggesting they form two distinct subsets. If ganciclovir was

withdrawn, tumour re-growth occurred after a further 2 weeks, suggesting TEM reconstitution was required (159).

TEMs have been identified in a variety of malignancies including lung, kidney, colon and pancreas (165), typically in the peri-vascular space (158). Circulating Tie-2 monocytes are present in patients with neuroendocrine cancer (158). Tie-2 expression is up-regulated with an enhanced function in these circulating cells (166). The presence of Ang-2 or hypoxia altered the secretory pattern of Tie-2 monocytes with suppression of IL-2 (anti-angiogenic) and IL-12 TNF-α (pro-apoptotic) (165).

1.8.2.5 Targeting Tie-2 Monocytes

Tie-2 monocytes have been considered for tumour targeting due to their ability to migrate across tissues. De Palma et al labeled TEMs with the gene for Interferon-α and obtained responses in orthotopic human gliomas and spontaneous mouse mammary carcinomas (167). An increase in apoptosis, inhibition of angiogenesis and an improved recruitment and activation of immune cells was seen. The agent was well tolerated with no myelotoxicity or impaired wound healing (167).

1.8.2.6 Tie-2 Monocytes as a Biomarker

Limited data exists on the efficacy of Tie-2 expressing monocytes as a biomarker, either for tumours or for agents such as the VDA's. Lee et al considered marrow Tie-2 and found it was a prognostic factor for survival in acute myelogenous leukaemia (168).

After a procedure such as TAE, intense hypoxia occurs, with rebound angiogenesis. If release of angiopoietin-2 is stimulated this may attract TEMs. Conversely the blood level of these monocytes may reflect the tumour volume.

1.8.3 Endothelial Progenitor Cells

Endothelial progenitor cells are bone-marrow derived cells circulating in the peripheral blood that appear to have a stem-cell phenotype. They have the ability to migrate, form primitive tubes and adhere to substratum (169) and have a high proliferation rate with the ability to form colonies. EPCs appear to be attracted to areas of neo-vascularisation guided by angiogenic cytokines, where they differentiate into endothelial cells before incorporation into the growing vessel. They were first identified by Asahara et al (170, 171) who demonstrated CD34+ve cells in the peripheral blood that could generate mature endothelial cells in vitro and in vivo (171). Their primary role appears to be in embryogenesis and vasculogenesis, considerable controversy exists as to their role in cancer.

In addition to EPCs, circulating endothelial cells (CEC's) are elevated in cancers and fall in responding disease (172). These are cells shed from tumour-associated endothelium rather than recruited from bone-marrow (173) and are differentiated endothelial cells. In patients with gender mismatched bone marrow transplants 95% of circulating endothelial cells were of vessel origin and only 5-6% of bone-marrow origin. Cells of bone-marrow origin could be expanded 1000-fold when cultured in vitro and are thought to represent EPCs (174).

1.8.3.1 Defining endothelial progenitor cells and circulating endothelial cells

Culture assays remain the gold standard to define a cell as an EPC. This method is not always practicable. Instead cell surface markers can be used with multi-parametric flow cytometry. Unfortunately no cell marker exists that defines an EPC with individual markers present on a wide range of cell lineages. Asahara et al (171) used the combination of CD34 and VEGFR2 co-expression to define an EPC. A variety of other cell markers have also been considered and no current standard definition exists of what constitutes an EPC. Different pre-enrichment steps and gating strategies have also been attempted. The variety of techniques makes it difficult to compare research papers as it is not clear the same cell population is being considered. It may be that different sub-populations of EPCs exist with differing characteristics and cell surface markers.

Table 1.3 Defining endothelial progenitor cells
Table adapted from one by Pathak et al (175)

Surface Marker	Endothelial progenitor cell	Circulating endothelial cell
VEGFR1	-	+
VEGFR2	+	+
VEGFR3	+	
FGFR1	+	
Tie-2	+	+
VE-Cadherin (CD144)	+	+
E-Selectin	+/-	+
CD34	+	Dim
CD31	+	+
CD133	+	-
cKit	+	+
CD13		+
vWF	-	
CXCR-4	+	+
CD146	+	+
CD45	Dim	-

+ = expression present; - = expression absent,; blank means no documentation
vWF= von Willebrand Factor; CXCR-4 = chemokine receptor-4, FGFR1 = fibroblast growth receptor-1, VE-Cadherin = Vascular endothelial cadherin

As EPCs develop from bone marrow to full differentiation their markers change. Some aspects of this pathway are known. EPCs straight after release into the circulation express CD133, CD34 and VEGFR2. As they mature into circulating EPC's they lose the marker CD133 (which is also expressed on haematopoietic stem cells) and may express mature endothelial markers (176).

CD34 is present on both immature and mature EPCs so cannot differentiate between them as is VEGFR2. CD146 is an endothelial cell marker (177) but is also present on some mesenchymal cells and a population of activated lymphocytes (178). The haematopoietic marker, CD45, is considered low or negative in EPCs.

1.8.3.2 Numbers in circulation

The number of circulating EPCs found varies widely depending on the technique used. With flow cytometry values are typically 70-210 cells/mL of blood (using CD34/133/VEGFR2) (179). With cell culture, higher numbers can be generated (180).

EPCs can be cultured by re-plating methods with colonies (CFU-ECs) appearing as round cells in the centre. Cells are plated on fibronectin-coated plates with tissue culture medium enriched with endothelial growth supplements. A pre-plating method to remove monocytes and haematopoietic cells can be done prior to plating to avoid contamination of the EPC culture. Colonies are seen as cell masses with central round cells and spindle shaped cells in the periphery. They are reported to express VEGFR2, CD31 and Tie-2 (181). Up to 10^5 cells can be generated by this method (182).

Peripheral blood mononuclear cells (PBMNC) can also be cultured on fibronectin plates with angiogenic cytokines for 4-6 days. After that time point

non-adherent cells are removed. The adherent cells are considered to be circulating angiogenic cells (CAC) and can enhance angiogenesis in vivo. They express endothelial markers such as CD31, vWF (von-Willebrand Factor) and Tie-2 and possess similar in-vitro characteristics but do not form the characteristic colonies as seen above (183). Concern exists that these CACs and CFU-EC groups may represent monocytes/macrophages rather than true EPCs as they may express colony stimulating factor-1 and can phagocytose *Escherichia coli* (184).

The different types of cell are summarized in Table 1.4.

Table 1.4 Potential endothelial angiogenic cells

Cell Type	Profile	Origin	Morphology
EPC	CD31/CD34/CD133/c-kit/CXCR2/VEGFR2	Bone marrow	Immature PB cells. 20μm
CFU-EC	CD31/CD34/Tie-2/VEGFR2	Culture	Colony formation
CAC	CD31/vWF/Tie-2/VE-cadherin	Culture	No colony formation
ECFC	CD31/CD36/Tie-2/VEGFR2/VE-cadherin/vWF	Culture	Cobblestone colonies

1.8.3.3 Endothelial Progenitor cells and cancer

Bone-marrow derived endothelial cells are present in tumour-associated blood vessels. They can restore impaired angiogenesis and are associated with tumour progression and the development of metastasis. They are elevated in cancer patients and have been considered as a potential biomarker for angiogenesis (173, 185, 186). Some EPCs remain in the walls of human umbilical veins,

representing a store of stem cells. If stimulated they can then differentiate into endothelial cells or be recruited by cancer cells (187, 188).

1.8.3.4 Incorporation into tumour-associated vasculature

Incorporation of bone-marrow derived endothelial cells into blood vessels has been demonstrated in tumour bearing mice (170). The degree of incorporation in animal models varies between different tumour types, grades, involved organs and mouse strains (189) and may vary with tumour growth rate and degree of hypoxia.

If immuno-deficient mice are transplanted with human cancer cell lines the percentage of bone-marrow derived cells in tumour endothelium varied from non-detectable (163), 10-20% (190) and up to 50% (191). Duda et al demonstrated that EPC-derived vessels were only 1.5% in mammary fat pad breast cancers but 58% in a mammary model of brain carcinoma metastases (192). EPCs preferentially localize to the tumour periphery in animal models where blood vessel density was highest (193). In HCC they are found in adjacent cirrhotic tissue to the tumour possibly under the influence of HIF-1α (194).

The role of EPCs in the formation of tumour endothelium remains controversial (195, 196) and their role may vary between different tumour types and at different developmental stages. For example Rajantie et al used multi-channel laser scanning confocal microscopy in mice to study purported EPCs and found that the cells remained in the peri-endothelium and resembled pericytes rather than true EPCs (197, 198).

1.8.3.5 EPC incorporation and stage of vessel development

EPC contribution to tumour vasculature changes over time. Mathematical models show the proportion of tumour endothelium derived from EPCs rising with increasing tumour size so that a 0.5mm tumour has 2% EPC derived, but 25% at 1mm and 40% at 1.5mm (188). This finding was confirmed in animal models (199, 200), reaching 38% in mice with insulinomas. The more advanced cancers appeared more able to recruit bone-marrow derived cells.

Although EPC levels are thought to increase with advancing disease, other models consider them most influential in early and late stage disease (201). Studies with low levels of EPCs are most often in untreated tumours at neither the early or late stage (178). It has been hypothesized that the role of EPCs is to generate a transient response to changed circumstances, such as in initial relapse post-therapy (178). They appear to rise after chemotherapeutics such as taxanes (202) and post-VDAs when they home to the viable tumour rim (202, 203), and may enhance tumour growth.

1.8.3.6 Enhancing tumour angiogenesis

EPC administration restored angiogenesis in angiogenic-defective (with impaired endothelial sprouting) Id-mutant mice with lymphoma (199). In these mice EPCs contributed 90% of tumour endothelium. If mice lacked placental growth factor, bone-marrow derived cells could also restore VEGF-driven angiogenesis (204). Gao et al implanted syngeneic mice, whose bone-marrow cells expressed green fluorescent protein, with Lewis lung cancer cells expressing red fluorescent protein (205). Bone-marrow derived cells were 12.7% of total endothelial cells. If EPC recruitment was prevented by suppressing the transcription factor Id, neo-vascularisation was reduced, there was a decrease in metastases and an increase in survival (205).

1.8.3.7 The stimulus to EPC release

EPC recruitment is thought to be secondary to hypoxia and the release of HIF-1α (206). Other factors considered likely to be involved are VEGF, GM-CSF (Granulocyte macrophage-colony stimulating factor), SDF-1 (stromal cell derived factor-1) and platelet-derived growth factor (207) as well as the soluble KIT ligand which induces proliferation and migration in the bone marrow (208). Exogenous VEGF stimulates a rise in circulating EPCs within 24 hours (209) possibly via activation of MMP-9 or up-regulation of stromal cell derived factor-1 (SDF-1) or CXCR4, which is chemotactic for EPCs recruiting them to areas of neo-vascularisation (176). Cell homing is enhanced by SDF-1 injection (in the presence of VEGF) and reduced by blockade of CXCR4 (210).

A dose-dependent increase in the number of functional EPCs has been seen post administration of recombinant human erythropoietin (rHuEPO) both in vitro and in human blood (211). In addition the level of circulating EPCs correlates with serum erythropoietin (212), plasma VEGFR2 and plasma interleukin-8 (213). EPC migration is stimulated by PDGF-1 (Platelet derived growth factor), Ang-1 (429), PIGF, nitric oxide, statins, exercise and oestrogens whereas a high CRP (C-reactive protein) or TNF-α is associated with a lower level (176).

1.8.3.8 EPCs as biomarkers

Mouse models show correlation between tumour size and circulating EPCs (214). Administering cyclophosphamide to a mouse produced short term EPC suppression followed by a rapid increase. If a metronomic schedule was used EPCs remained suppressed. Endostatin and VEGFR2 antibodies both led to a decrease in EPCs (202) that correlated with tumour response (186) as did bevacizumab in rectal cancer (176).

EPCs have been studied as a method to determine the optimal biological dose of a blocking antibody to VEGFR2 (178) with the dose defined as that producing the lowest number of viable EPCs. This method gave approximately the same result as that from dose-response studies (800μg per mouse versus 800-1200μg respectively). The fall in EPCs did not correlate with tumour size or tumour reduction. Optimal biological doses have been determined for other anti-angiogenic therapies including metronomic chemotherapy schedules (178).

1.8.3.9 Clinical data

The contribution of EPCs to human tumour vasculature remains uncertain. Peters et al studied 6 patients who received a sex mismatched bone marrow transplant and then developed cancer. The contribution of bone-marrow cells in tumour vasculature ranged from 1% in a head and neck tumour to 12% in a lymphoma with an average of 4.9% (215). No fusion between bone-marrow derived and existing cells was seen by FISH (Fluorescent in situ hybridization) analysis.

Table 1.5 EPC levels in human malignancy

Table adapted from Ding et al (188).

Tumour type	EPC levels	Correlated with
Lymphoma	Circulating and biopsy derived EPCs	Response to therapy and disease activity Igreja et al (432)
Myeloma	Circulating EPCs	Response to therapy and disease activity Zhang et al (433)
Acute myeloid leukaemia	Circulating endothelial like cells	Disease activity Wierzbowska et al (434)
Breast cancer	Circulating Tie-2, mRNA, circulating EPCs	Tumour size; Naik et al (435) Disease activity; Sussman et al (436) Response to therapy and survival Shirakawa et al (437)
Colorectal cancer	Circulating EPCs	Treatment response and predict recurrence Lin et al (438)
HCC	CFU scores, Circulating EPCs and tissue-derived EPCs	Recurrence time; Ho et al (439) VEGF and prognostic markers; Yu et al (409)
NSCLC	Circulating EPCs	Survival, response to therapy Dome et al (440) Disease activity (441)

The level of circulating endothelial progenitor cells has been correlated with prognosis in NSCLC with a level of > 1000/ml in peripheral blood associated with a poorer survival (216). Responding disease was associated with a greater fall in EPCs than in non-responders. In hepatocellular cancer patients had higher levels of EPCs (using colony-forming scores) than control groups without liver disease or with cirrhosis alone (143). Patients with un-resectable disease had higher levels than patients who were suitable for surgery, and in patients undergoing potentially curative surgery, higher levels were prognostic for relapse within

one year. In breast cancer levels were increased and acted as a prognostic marker (217). Levels of CD133, Tie-2 and VE-cadherin mRNA were elevated in patients with infiltrating breast cancer (217). On receiving chemotherapy a 28% decrease in EPC levels could be seen (218).

If EPCs levels do reflect angiogenesis they may be good biomarkers for drugs with anti-angiogenic properties including metronomic chemotherapy. In rectal cancer a single infusion of bevacizumab led to a decrease in peripheral blood EPCs (219). A reduction in circulating EPCs post anti-angiogenic therapies has been demonstrated in lymphoma (220), myeloma (221) and AML (222). Non-responding patients do not show a fall in circulating EPCs.

1.8.4 Hypothesis Four

Our hypothesis was that the acute hypoxia induced by vascular shut down would lead to an increase in angiogenic cytokines. Tie-2 expressing monocytes would be recruited from bone marrow either secondary to hypoxia or to the hypoxia-induced increase in the angiogenic cytokines. Tie-2 monocytes levels would increase in the circulation and target tumour, promoting further angiogenesis. Monocytes expressing Tie-2 would be anticipated to rise in the circulation post vascular shut down. Tie-2 monocytes may therefore have potential as a biomarker for the VDA's.

In Chapter Five I report a preliminary study to evaluate the feasibility of assessing the level of Tie-2 monocytes in the peripheral blood as a biomarker that could in future be used to assess the VDA agents.

Endothelial progenitor cells are known to be recruited to tumours under the influence of the angiogenic cytokines and may subsequently enhance angiogenesis. Our hypothesis is their levels will rise in the circulation post TAE

secondary to hypoxia and ischaemic necrosis. Their level in the circulation may have potential as a biomarker.

In Chapter Five I report our preliminary study considering the EPC's as a potential biomarker for the vascular shut down induced by TAE. No standard methodology currently exists for identifying EPC's by flow cytometry, therefore 3 separate methods were explored to consider their respective feasibilities.

Chapter Two ^{131}I-CHT25 for CD25 expressing lymphoma

This Chapter will report the data from our phase I clinical trial assessing the initial use of radioimmunotherapy in CD25 expressing lymphomas (predominantly Hodgkins' lymphoma). Before presenting the data I shall consider the chosen antigen in greater detail and previous attempts at targeting it with different molecules.

2.1 Interleukin-2 (IL-2) and its receptor (CD25)

The IL-2 molecule is a 15 kDa globular protein, pivotal to the activation and proliferation of T-lymphocytes (223). The binding of effector T-cells to their antigens leads to secretion of IL-2 and up-regulation of IL-2R (Interleukin-2 Receptor) on the cell surface. A positive feedback loop then magnifies clonal expansion. The cell with the greatest concentration of IL-2R is the T-regulatory cell and IL-2 has a significant role in the maintenance of tolerance.

2.1.1 The interleukin-2 receptor (CD25)

The IL-2 receptor has 3 subunits: see Table 2.1. The alpha subunit is restricted to the IL2R whereas the beta and gamma subunits are common to many cytokines.

Table 2.1: Subunits of the Interleukin Receptor

Subunit	**Alt names**	**Weight (kDa)**	**Gene found**
Alpha	CD25 or Tac	55	Chromosome 10
Beta	CD122	75	Chromosome 22
Gamma	CD132	64	X Chromosome

The beta and gamma units are required for signalling and receptor complex formation. Their ubiquity means they are less useful as targets. The alpha subunit is essential for a high-affinity binding site (223). Subunits can bind individually but at a lower affinity. Loss of the alpha subunit leads to abnormalities of T-cell regulation with an expansion of autoreactive T-cell clones, tissue infiltration by T-cells, atrophy and inflammation (224). Episodic suppression does not appear to cause this dysregulation (224).

2.2 CD25 expressing lymphomas

CD25 is expressed on many lymphoma cells but only a small number of normal cells (activated lymphocytes and T-regulatory cells) that represent < 5% of the normal T-lymphocyte population at any one time. It is expressed at extremely high density (10^3 to 10^4 per cell) in HTLV-1 (human T-cell Lymphotropic virus) associated adult T cell leukaemia / lymphoma (ATLL) where it is involved in an important growth pathway (225).

CD25 is expressed to a variable extent on other lymphomas. It is highly expressed (>75%) in hairy cell leukaemia, peripheral T-cell lymphomas, and Hodgkin's lymphoma (75-90%). In addition it is present on approximately 50% of T-cell lymphomas (mixed) and cutaneous T-cell lymphomas, B-cell (intermediate and high grade) and 50% chronic lymphocytic leukaemia (226, 227).

The first antibody to CD25 was named anti-tac as it bound to active T lymphocytes (228). A humanized anti-tac has since been developed (daclizumab) and a chimeric antibody to a separate epitope (basiliximab). Patients with any CD25-expressing lymphoma were eligible to enter our study. In the following section I shall briefly discuss some of these disease entities.

2.2.1 Hodgkin's lymphoma (HL)

Hodgkin's lymphoma (HL) cells (Hodgkin or Reed-Sternberg cells) appear to derive from B-cells at different developmental stages (229). They make up < 1% of tumour bulk; the rest appears to be reactive lymphocytes and other immune cells. Current treatment strategies (EBRT and combination chemotherapy) cure approximately 80% of patients. In relapsed disease high–dose therapy with ASCT can cure approximately an additional 50% (230). Allogeneic transplant has not improved survival and has a high mortality rate (231).

2.2.1.1 Targeted therapy of HL

A variety of new targeted approaches have been attempted including the use of antibodies, modulation of transcription pathways and anti-EBV (Epstein Barr virus) agents. The first RIT trials used polyclonal anti-ferritin antibodies conjugated with ^{131}I. A phase II clinical trial treated 38 patients and saw a partial response rate of 40% with symptomatic benefit in 77% (232).

CD30 is highly expressed in HL and relatively specific so a suitable target. Both the murine (Ber-H2) and chimeric (SGN-30) unlabeled antibodies to CD30 had no responses in patients with HL although 4 patients had stable disease for 6-16 months and 1 patient with anaplastic large cell lymphoma had a CR (Complete response (233). A humanized antibody showed limited activity with 5 of 72 patients showing a response in a dose escalation phase I/II study but had no clear dose response relationship (234). Studies of this combination with chemotherapy are continuing.

The chimeric anti-CD30 antibody, SGN-30, has been attached to monomethyl auristatin E, a tubulin inhibitor. In an animal model of HL it appeared potent and selective (235) with a synergistic effect with chemotherapy (236) but clinical

studies are awaited. Conjugating an anti-CD30 antibody with a radionuclide ^{131}I, produced a response rate of 6 out of 22 patients (237).

2.2.2 T-cell lymphomas

T-cell lymphomas make up approximately 12% of lymphomas (238) and vary greatly in their aggressiveness and response to therapy. Their distribution depends on the prevalence of viruses such as HTLV-1 and EBV (239). A variety of T-cell lymphomas express CD25 including angioimmunoblastic lymphoma (AITL), peripheral T-cell lymphoma, unspecified (PTCL-u) and adult T-cell leukaemia/lymphoma (ATLL).

2.2.2.1 Adult T-cell leukaemia / lymphoma (ATLL)

ATLL results from infection with the retrovirus HTLV-1. Four subtypes exist based on signs, symptoms and prognosis: an acute leukaemia type, nodal lymphoma type, chronic leukaemia type or a smouldering course (240). It initially responds well to chemotherapy but quickly relapses (240). High dose interferon-α combined with zidovudine appeared promising in 2 small studies (241, 242) as initial therapy or maintenance.

Initial studies of targeted agents used the unlabeled anti-Tac antibody and demonstrated a modest benefit with responses lasting 1-8+ months (243). Anti-Tac was then radiolabeled with ^{90}Y and given to 18 patients (9 in the dose-escalation phase). Of 16 evaluable patients 9 responded. Mean response duration was 9.2 months for the 7 patients in PR (Partial response). Of the 2 in CR, one died of leukaemia at 36 months and the other remained relapse-free at 3 years (244).

2.3 CHT-25 clinical trial

The CHT25 clinical trial was a single centre, open label, phase I study using the monoclonal chimeric antibody, basiliximab, to target CD25. The antibody was conjugated to ^{131}I to augment cytotoxicity. The study was primarily dose-finding but was designed to gain further information about toxicity and response.

It was estimated that trial recruitment would begin in March 2000 and complete by 2003. However recruitment started in December 2002 and 9 patients were treated by July 2004. The trial was then suspended due to regulatory issues arising from the introduction of the EU Clinical Trials Directive and only re-opened in June 2006 with recruitment completed in June 2007. It was approved by the Central Institutional Review Board (CIRB) and the Royal Free Hospital Local Ethics Committee.

2.3.1 Hypothesis

The trial hypothesis was that the RIT product CHT25 would selectively target the malignant cells in CD25-expressing HL and T-cell lymphoma leading to tumour responses with a low toxicity. As CHT25 is a chimeric antibody, re-treatment should be possible without high rates of HAMA /HACA formation.

2.3.2 Trial objectives

The primary objectives were to determine the toxicity profile of CHT25, define the dose-limiting toxicity (DLT) and the maximum tolerated dose (MTD), investigate the localisation, biodistribution and dosimetry of CHT25 in tumour and normal tissues and determine the pharmacokinetic profile of the RIC. The secondary objectives were to assess preliminary evidence of efficacy and immunogenicity.

2.4 Methods

2.4.1 The investigational product

CHT25 is a chimeric antibody conjugated to ^{131}I. The antibody, basiliximab, was derived from the mouse antibody RFT5$\gamma 2\alpha$. It was produced in vitro by continuous culture fermentation of a murine-myeloma cell line which had previously been transfected with plasmid borne recombinant gene constructs coding for its murine variable and human constant regions (245). CHT25 binds to the alpha subunit of the IL-2 receptor with a similar affinity to IL-2 itself. Cross-reactivity to normal tissues was excluded by immunoperoxidase and immunofluorescent studies with normal tissues (Dr Amlot – personal communication).

Basiliximab is a licensed drug to prevent organ rejection in kidney transplant patients and has been extensively studied. Toxicology on the antibody used rhesus monkeys and concluded the agent was well tolerated with a terminal half-life of 5.5 days and 3-4 days on repeat exposure. Human studies at doses of 20mg administered intravenously revealed no severe reactions, with a longer half-life of 13.1 days (range 7-23 days) (245, 246). In a phase I study of 24 patients and 144 infusions (6 administrations per patient) there was no evidence of HAMA or HACA antibody generation using ELISA (Enzyme linked immunosorbent assay) or FACS (fluorescence activated cell sorting) inhibition analysis and there was no reduction in half-life supporting the lack of an immune reaction (246).

A phase III study of 190 patients compared basiliximab to placebo in the prevention of transplant rejection (246). A 32% reduction in the number of acute cellular rejection episodes was seen in the basiliximab arm with no increase in side effects. Basiliximab is licensed for the prevention of rejection under the brand name Simulect.

2.4.2 Pharmaceutical

Basiliximab was supplied by Novartis as 10mg vials lyophilised for reconstitution and stored at 2-8°C. The ^{131}I was ordered via the Department of Medical Physics and supplied by Amersham or MDS Nordion as a sodium iodide solution in standard vials. It was stored in the radioactivity storage area at room temperature.

2.4.3 Reconstitution and relabeling

A qualified radio-pharmacist performed the labelling process using the N-bromo-succinamide/ L-tyrosine technique (247) at the Royal Free Hospital.

2.4.4 Selection of starting dose and schedule

Basiliximab was given at a fixed intravenous dose of 10mg as a targeting dose to tumour cells that was previously well tolerated (246). The initial administered activity of ^{131}I was chosen at 370MBq/m^2 on the basis of dosimetry calculations from previous work (unpublished) with the chimeric B72.3 anti-CEA antibody. These calculations assumed the CHT25 distribution in tissues that do not express CD25 would be the same as the anti-CEA antibody in tissues that do not express CEA.

The intended schedule for the first 9 patients is shown in Table 2.2. Each cohort consisted of 3 patients. A 7 day window was required before treating an additional patient at the same dose level, and 4 weeks for escalation to the next level.

Table 2.2 Intended schedule			
No of Patients	**Antibody Treatment (mg)**	**Isotope treatment (MBq/m^2)**	**Dose increased by**
3	10mg	370	-
3	10mg	740	2
3	10mg	1480	2
3	10mg	2220	1.5
3	10mg	2960	1.3

The amount of ^{131}I labelled to the CHT25 antibody varied slightly as it is not possible to label doses with absolute accuracy.

2.4.5 Repeat dosing

Repeat dosing was permitted either at an escalated dose or the same dose if these criteria were met:

- A minimum of 4 weeks had elapsed to identify toxicities.
- Evidence of localisation of CHT25 to tumour; tumour uptake had to be > 3% injected dose/kg.
- No DLT's were observed at the current level; infusion of stem cells or bone marrow cells were not required
- All the eligibility criteria (inclusion and exclusion criteria were still met)
- Any treatment related toxicities had resolved

Patients who had escalation of their treatment could be included in a second cohort at the higher level.

2.4.6 MTD-finding phase

If one of three patients in a cohort experienced a DLT the cohort was expanded to 6. If in that expanded cohort no additional DLT's were seen, dose escalation could continue unless the DLT was a patient death (drug-related). If 2 patients had a DLT or 1 patient had a drug-related death the treatment would de-escalate to a lower level (the previous dose level or lower) to define the MTD.

After the first 9 patients were treated an interim analysis considered the available evidence on efficacy and toxicity. It was decided to explore further injected activity levels between 740 MBq/m2 and 1480 MBq/m2 with additional cohorts investigated at both 1200 MBq/m2 and 1480 MBq/m2.

2.4.7 Administration of agent

Administration of CHT25 was over 40 minutes via a 1m infusion line and 3-way tap with a lead shield for the RIC. The initial infusion rate was 6mg/hour, increased to 24mg/hour if no side effects occurred.

2.4.8 Inclusion and exclusion criteria

2.4.8.1 Inclusion Criteria

- A histologically proven lymphoma that expressed CD25 on ≥ 50% of malignant cells or the surrounding stromal cells in HL.
- Refractory or relapsed disease post standard therapy or medically unfit for standard therapy
- Measurable disease on imaging; whether X-ray, ultrasound, CT, MRI (Magnetic Resonance Imaging) or PET.
- An age greater than 18 years
- A life-expectancy ≥ 3 months without therapy
- A WHO (World Health Organisation performance status ≤ 2;
- In the first 1-9 patients or if an injected activity of $^{131}I \geq 1480 MBq/m^2$ was given a stem cell harvest was required (minimum $2 \times 10^6/L$ CD34 positive cells) or a bone marrow harvest (minimum $1 \times 10^6/L$ CD34 positive cells) to allow PBSCT (Peripheral blood stem cell transplant)
- Adequate organ function; including
    - Neutrophil count > $1.5 \times 10^9/L$.
    - Haemoglobin > 10g/dL.
    - Platelet count > $100 \times 10^9/L$
    - Plasma creatinine < 150µmol/l; EDTA (Ethylenediamine tetraacetic acid) or urine creatinine clearance >50ml/min.
    - Plasma bilirubin < 30 µmol/l, alanine transaminase (ALT) and aspartate aminotransferase (AST) < 2 x upper limit of normal (or 5 x if liver metastases).
    - International normalized ratio (INR)> 1.5.
    - Normal thyroid function or stable on treatment.
    - Adequate cardiac function; ejection fraction > 50% on MUGA (Multi-gated acquisition scan) or on echocardiography.

- Be able to provide written informed consent; be capable of co-operating with therapy and follow up.

2.4.8.2 Exclusion criteria

- No previous lymphoma therapy within the previous 4 weeks (excluding a stable dose of steroids or palliative radiotherapy to a non-target lesion); for nitrosureas or mitomycin C the period was 6 weeks.
- No bone marrow involvement by lymphoma > 25% on biopsy.
- Pregnancy, lactation or women of child-bearing age in whom pregnancy could not be excluded, those of either sex of child-bearing age unable to use adequate contraception for the study period.
- Serious non-malignant systemic disease, active uncontrolled infection, severe respiratory disease or serologically positive for hepatitis B, C or Human Immunodeficiency virus (HIV).
- A positive test for HAMA to CHT25.
- Any other medical condition that made them unsuitable in the opinion of the investigator.

2.4.9 Pre-trial investigations

The pre-treatment investigations required are detailed in Table 2.3. Patients were followed up for a minimum of 8 weeks or until all drug related toxicity had resolved. Potassium Iodide was administered initially at 100mg three times a day for 3 days, reducing to 50mg bd up to day 11. Patients remained in-patients with appropriate radiation-protection shielding until safe for discharge. Patients were assessed daily as inpatients with haematology, biochemistry, symptom history, vital signs, PK (Pharmacokinetic) analysis and γ-camera imaging recorded. The follow up protocol is summarized in Table 2.4.

Table 2.3 Pre-trial investigations	
Date pre-therapy	**Nature of investigation**
Within 12 weeks	Blood taken for HAMA
Within 4 weeks (preferably within 2)	Baseline evaluations for tumour extent (CT/PET)
Within 2 weeks	Informed consent Chest X-ray, ECG and bone marrow examination, EDTA or 24-urine collection and ECHO or MUGA
Within 1 week	Haematological and biochemical indices Medical history and examination Documentation of WHO status Registration with Cancer Research UK Drug Development Office

Table 2.4 Follow-up protocol	
Follow-up protocol post discharge	**Nature of investigation**
Twice-weekly	Haematology and Biochemistry
Weekly	Clinical assessment including physical examination (with WHO status) T-lymphocyte assessment (first 9 patients only)
Day 8, 15 and 22	Urinalysis (blood, protein, pH, glucose)
Day 15, 29, 43 and 57	Plasma clotting screen
Day 25 and 53	FDG-PET scan
Day 22, 43 (if planned to re-treat) and 57	HAMA
Day 29 and 57	Tumour Imaging assessments ECG, creatinine clearance, Thyroid function, and MUGA or ECHO scan

The blood and urine tests included in the above definition were

- Haematology; Haemoglobin, white cell count, neutrophils, lymphocytes and platelets.
- Biochemistry; Sodium, potassium, urea, creatinine, urate, total protein, bilirubin, AST, ALT, Alkaline phosphatase, glucose, GGT (gamma-gutanyl transferase)
- Clotting screen; PT (prothrombin time) APPT (activated partial thromboplastin time)
- Thyroid function tests; Plasma T4 and TSH (Thyroid stimulating hormone).

2.4.10 Toxicity

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (version 2). The worst grade in each cycle was documented.

2.4.10.1 Definition of a dose-limiting toxicity (DLT)

A DLT was defined as a toxic event almost certainly or probably drug related (see Table 2.5). In the dose escalation phase bone marrow and stem cells were stored and could be re-infused if the neutrophil count fell below 0.2×10^9/l for > 2 days. In the MTD-finding phase a lower non-myeloablative administered activity was given. The definition of a haematological DLT was changed to reflect this.

Table 2.5 Dose-limiting toxicity

System	Escalation phase (9 patients)	MTD finding (6 patients)
Bone marrow	Grade 4 febrile neutropenia	Grade 4 neutropenia not responding to therapy in 10 days Grade 3 or 4 neutropenia associated with fever / infection Grade 4 thrombocytopenia either lasting more than 5 days or with bleeding or requiring transfusion
Nervous system	Grade 2 or greater neurotoxicity	
Other body organs	Any toxicity grade 3 or higher excluding Grade 3 nausea and Grade 3 / 4 vomiting and / or diarrhoea in patients not optimally treated	
Whole body	Drug related death	

2.4.10.2 Definition of Maximum Tolerated Dose (MTD)

The MTD was defined as the dose above that at which either a single drug related death occurred or where more than 30% of patients suffered dose limiting toxicity.

2.4.11 Response assessment

Tumour response was determined using CT and FDG (fluorodeoxyglucose)-PET. These two methods were combined using the Updated International Working Group Response Criteria (248, 249) for lymphoma (see Table 2.6). Tumour assessments were performed 2 weeks prior to trial entry and then every 4 weeks. Complete or partial responses were confirmed by a second study 4 weeks later.

2.4.11.1 CT

Lesions were assessed according to WHO criteria. Bi-dimensional lesions had their longest diameter and the perpendicular measurement at the widest point recorded for a maximum of 10 lesions.

2.4.11.2 FDG-PET

Imaging was performed using an ADAC Vertex Plus Dual Head Co-incidence camera hybrid SPECT/PET camera (Phillips-ADAC, Eindhoven, Netherlands or a GE Discovery LS PET-CT (GE Healthcare, Amersham, UK). Two patients had diabetes and were unable to tolerate the scanning time on the hybrid camera, their ^{18}FDG-PET scans were completed with the GE Discovery LS PET-CT. The images were assessed visually and with a semi-automated region growing program. Visual assessment was by a Consultant in nuclear medicine blinded as

to patient identity and scan order to attempt to reduce the subjectivity and bias inherent in this approach.

Table 2.6 Cheson criteria

Response	Definition	Nodal mass	Organs	Bone marrow
CR Complete Response**	All evidence disease disappeared	Any size mass allowed if PET negative	Nodules disappear	Infiltrate disappeared on biopsy/IHC*
PR[+] Partial Response	Regression measurable disease / no new sites	>50% reduction in disease on CT / at least 1 site remains PET positive	As nodal	Irrelevant if positive prior to therapy
SD[++] Stable Disease	Failure to attain CR/PR	PET positive sites remain and no new sites on CT or PET. No change in size CT lesions	As nodal	
PD Progressive Disease	Any new lesion or increase by > 50% of previous lesions.	New lesions or a >50% increase in the size of more > than 1 node. Lesion PET positive.	>50% increase from nadir	New or recurrent involvement of bone marrow.

* Immunohistochemistry; **CR Complete response; [+] PR Partial response; [++] SD Stable disease
[&] PD Progressive disease

^{18}FDG uptake can be assessed semi-quantitatively by the SUV (standardized uptake value). The mean tissue radiotracer concentration is obtained from a region of interest (ROI) and corrected for injected activity. It is compared to a homogeneous body distribution of radiotracer (250). Problems with this method are in determining where to apply the ROI in the tissue of interest, it is unclear if the relevant value is the mean or the maximum count per voxel and SUV measurements only record tumour intensity with no account of shrinkage.
An alternative method pioneered in our department by Dr Alan Green and the Department of Medical Physics was a region growing program (251). This

program allows the inclusion of both intensity and tumour size to assess response. Initially a threshold value was determined, intensity values above the threshold were considered to be tumour and those lower were considered to be normal tissue. A seed-point was placed in an area of tumour by the operator. The placing was determined by visual inspection of tumour on PET images. The CT images were available for comparison but no co-registration was feasible during this study period.

From the seed-point placed the computer program grows the region outwards contiguously including any point that had a voxel above the predetermined threshold. An image is then produced of all the included data points. The computer program then re-calculates the image from the seed-point that the computer has identified as having the highest voxel count. The second calculation should ensure that all relevant voxels are included. It continues re-calculating from alternative seed-points until the results are identical indicating there are no further voxels uncounted that are above threshold. The computer then gives a numerical value including both voxel intensity and number of voxels. This method can then be repeated for a number of target lesions that can then be followed up during the study period.

The advantages of this method over using SUV's are that it includes both tumour shrinkage and a reduction in image intensity in assessing tumour response. It is also less operator-dependent. With SUV calculations the value can be altered by where the operator chooses to measure it from. With a region-growing program, wherever the operator places the original seed-point the value should be identical.

The disadvantages of this method are its current lack of clinical validation. It has not yet been validated in lymphoma so no clarity exists about what constitutes response or progressive disease. Green et al have validated it in solid tumours

where it was found to discriminate patients at 9-12 weeks successfully into those who went on to have a partial response and those with progressive disease (251) and clear distinction could be seen as early as 2-4 weeks. These studies have not been carried out in patients with lymphoma.

In addition any tumours that are situated close to a structure that has high FDG-PET uptake are problematic. The structures of concern classically are the heart and the bladder where voxels of interest can 'bridge' over into the anatomical structure. In addition if FDG-PET uptake is low, this method can show a lack of discrimination between low intensity disease and normal tissue. The method does not address other concerns with the SUV, namely ensuring scans are completed at identical time points post injection, with comparable glucose concentrations and at the same temperature.

2.4.12 SPECT and dosimetry data

Patients had Planar and SPECT imaging performed at 4, 24, 48, 72 and 96 hours using an ADAC Vertex dual headed gamma camera and reconstructed iteratively (OSEM (ordered subsets expectation maximization) algorithm) applying scatter and attenuation correction. ROI's were drawn on tumour and normal organs (heart, lung, liver, spleen and kidneys) and average radioactivity uptake was calculated using Olinda/XEM (Organ level internal dose assessment/ exponential modeling) (35) software.

2.4.13 Pharmacokinetics

Post administration of CHT25, 2.5ml of blood was taken in an EDTA tube at 1, 3, 6 and 24 hours post infusion and on days 2,3,6 and where possible day 9. Blood was stored until safe to analyse. Radioactivity was measured with a Packard Cobra 11th Series Auto gamma counter. Data was corrected for decay and

expressed as % injected dose/kg. Mono- or bi-exponential decay curves were modelled to the data and log linear regression used to calculate the half-lives.

2.4.14 Soluble Interleukin 2 receptor (sIL-2R)

The sIL-2R assay used a standard ELISA sIL-2R kit (Cellfree® Human). This kit used 50µl of serum previously stored at -80˚C. Control samples were prepared by serial dilutions of sIL-2R. 50µl of conjugate reagent was added and the plate incubated for 2 hours before the unbound reagent was washed off. 100µl of TMB (tetramethylbenzidine) substrate was added and the plate incubated for 30 minutes to allow enzymatic colour change before the stop solution was added. The colour intensity was read using a plate reader at 450nm subtracting 550nm to reach the final result. A curve was constructed using the control samples of known concentration and used to calculate the concentration in the serum. The value was expressed in pg/ml.

2.4.15 Immunogenicity

HAMA levels were assessed by ELISA pre-study and on days 43 and 57 with the murine antibody RFT5 and the non-specific isotype matched antibody RDFR2 as positive and negative controls respectively.

2.5 Results

2.5.1 Patient Characteristics

The CHT25 study opened for recruitment in December 2001 and completed in August 2007. In that period 15 patients were treated on study; 1 patient with HL was subsequently excluded when a second biopsy revealed dual pathology with a CD25 negative B-cell lymphoma. His results were not included. Of the 14 patients; 11 had HL and 3 had T-cell lymphoma (of which 2 had ATLL). 5 women and 9 men were treated. The median age was 42 years (range 27-70). The mean number of prior therapies was 4 (range 1-8). Full details are given in Table 2.7.

Table 2.7 Patient Characteristics	
Patient characteristics	**No patients**
Number of patients treated	14
Sex	
Male	9
Female	5
Age	
Median (range) in years	38 (27-70)
Tumour type	
Hodgkin's lymphoma	11
ATLL (adult T cell lymphoma/leukemia)	2
AITL (angioimmunoblastic T cell lymphoma)	1
Performance Status	
0	8
1	5
2	1
Ann Arbour Stage	
I	0
II	3
III	2
IV	9
B symptoms	
No	5
Yes	9
No of patients receiving 'x' prior regimens of chemotherapy	
1	1
2	2
3	4
4	3
5	3
8	1
Previous ASCT (autologous stem cell transplant	
No	5
Yes	9

2.5.2 Administered activity received

Table 2.8 details the administered activity given to each patient on study and the cumulative administered activity. In total 26 treatment courses were given. The first 9 patients followed an escalating schedule from 370MBq/m^2 to 2960MBq/m^2 with escalation allowed in the same patient.

The maximum number of cycles received by a single patient was 4 (patient 4). At the highest dose level a treatment related death occurred at 2960MBq/m^2. For safety reasons the next 2 patients had their initial injected activity reduced to 740MBq/m^2 where bone marrow suppression had not been significant. This reduction was aimed at excluding patients found to have a low bone marrow reserve from an excessive risk of toxicity.

After patient 9 a planned interim analysis was carried out. This considered that evidence of responses had been seen at lower doses that were unlikely to be myeloablative and (between 740MBq/m^2 and 1480MBq/m^2) recommended this lower range be investigated further. A dose cohort was therefore added at 1200MBq/m^2 with an additional 2 patients treated at 1480MBq/m^2. In this second MTD finding phase no dose escalation was allowed in individual patients to allow for a better discrimination between dose levels.

Table 2.8 Administered Activity					
Patient No	**Course 1 MBq/m^2 (MBq)**	**Course 2 MBq/m^2 (MBq)**	**Course 3 MBq/m^2 (MBq)**	**Course 4 MBq/m^2 (MBq)**	**Cumulative Activity MBq/m^2 (MBq)**
1	370 (663)				370 (663)
2	370 (573)				370 (573)
3	370 (659)	740 (1368)			1110 (2027)
4	740 (1027)	1480 (1858)	2220 (3480)	370(554)*	4810 (6919)
5	740 (1290)	1480 (2220)			2220 (3510)
6	740 (1104)				740 (1104)
7	1480 (2394)	2220 (3560)	2960 (4553)**		6660 (10507)
8	740 (1093)				740 (1093)
9	740 (1175)	1480 (2104)	2220 (3239)		4440 (6518)
10	1200 (2322)	1200 (2487)*			2400 (4809)
11	1200 (1604)	1200 (1642)			2400 (3246)
12	1200 (2666)	1200 (2650)			2400 (5316)
13	1480 (1999)				1480 (1999)
14	1480 (2620)				1480 (2620)

- * Given as a compassionate treatment as did not meet the re-treatment criteria
- ** Treatment related death at this administered activity

2.5.3 Toxicity

2.5.3.1 Haematological toxicity

Dose-limiting toxicity was haematological. Table 2.9 shows the nadir seen with an increasing injected activity. No grade 3 or 4 haematological toxicity was seen with the initial administered activity of 370MBq/m^2 but toxicity increased as the administered activity was escalated to 2960MBq/m^2. Patient 7 (2960MBq/m^2) developed prolonged myelosuppression and despite stem cell support, died 36 days after cycle 3 of *Pneumocystis jiroveci* pneumonia. De-escalation occurred to 1200MBq/m^2 which was tolerated well but further grade 4 haematological toxicity occurred at 1480MBq/m^2 with a median platelet nadir of 31 x 10^9/L (range 9-83) and neutrophil count of 1.31 x 10^9/L (range 0.9-7.5). Patients with Grade 4 neutropenia were treated with G-CSF (granulocyte colony stimulating factor) to count recovery. Blood transfusion was used to maintain haemoglobin between 12-14g/L for 4 weeks post administration to maximize radiation effect. Six patients required platelet transfusion at or above the MTD.

Table 2.9 Haematological Toxicity By administered activity – depth of nadir				
Administered activity (MBq/m^2)	**Haemoglobin nadir (grams/dl) (median/ range)**	**Neutrophil nadir ($x10^9/l$) (median/ range**	**Platelet nadir ($x10^9/l$) (median/ range)**	**Lymphocyte nadir ($x10^9/l$) (median/range)**
370 **3 treatments**	9.8 (8.4 – 11.4)	3 (2.7-3.7)	187 (74-195)	0.45 (0.31-0.96)
740 **6 treatments**	9.5 (9-11.5)	4.7 (1.3-11.3)	198 (60-384)	0.38 (0.19-1.55)
1200: 5 treatments* **3 patients**	8.8 (7.7-10.2)	1.3 (0.9-7.5)	31 (9-83)	0.2 (0.18-1.55)
1480 **6 treatments**	8.4 (7-10)	0.8 (0.1-7.8)	15 (8-150)	0.2 (0.1-0.58)
> 2220: 4 treatments* **3 patients**	8.5 (6.3-9.5)	1 (0.34-2.7)	13 (8-36)	0.105 (0.06-0.21)

The pattern of haematological toxicity with CHT25 was similar to that with other RIC's such as ^{131}I-T with a nadir approximately 6 weeks after therapy, resolving over a number of weeks. The depth and length of the nadir was dependent on the injected activity administered. Table 2.10 demonstrates this relationship.

Table 2.10 Haematological Toxicity By duration of nadir using CTCAE† 2					
Administered activity (MBq/M^2)	370	740	1200	1480	> 2220
Grade 3 Platelets (range)*	Nil	Nil	3-42 days	27-78 days	32-176 days
Neutrophil count Grade 4 (range)	Nil	Nil	Nil	2-30+[a] days	36[b] days

*Grade 3 toxicity was considered as grade 4 thrombocytopenia was treated with platelet infusion so nadir length cannot be judged. [a]One patient had allogeneic transplant before count recovery. [b]Treatment related death occurred at 36 days

† Common toxicity criteria adverse events version 2

To demonstrate the course of haematological toxicity, two patient's counts are documented. Figure 2.1 demonstrates the effect of treatment at $1200MBq/m^2$ with a limited nadir particularly affecting the platelet count with good recovery.

Figure 2.2 demonstrates the effect of receiving a higher injected activity (2220MBq/m2) with a subsequent prolonged and lower nadir count.

Figure 2.1 Patient 10

Level of platelet count (x10^9/L), neutrophil count (x10^9/L) and haemoglobin (g/dL) post RIC administration (days)

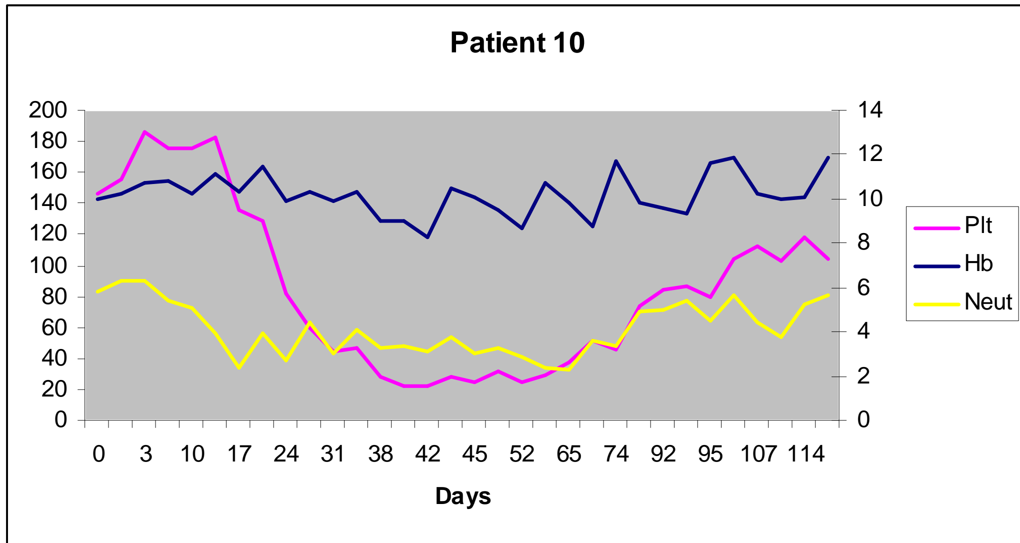
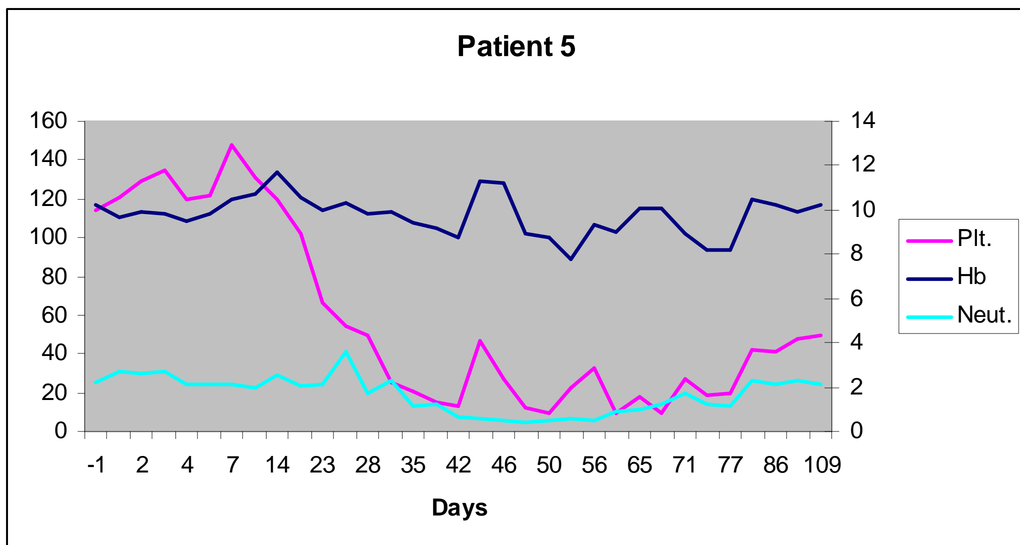


Figure 2.2 Patient 5

Level of platelet count (x10^9/L), neutrophil count (x10^9/L) and haemoglobin (g/dL) post RIC administration (days)



2.5.3.2 Non-haematological toxicity

Non-haematological toxicity was generally mild. The commonest was fatigue and a mild elevation of the liver enzymes. A high level of non-neutropenic infection was noted; whether this was secondary to an immunosuppressive effect of the agent or was the result of the trial patient population (heavily pre-treated and immunocompromised) is not clear. There was no evidence of viral reactivation, as seen with known immunosuppressive regimens. The details of all toxicities encountered considered to be certainly, probably or possibly drug-related are documented in Table 2.11.

Table 2.11 Non-haematological toxicity

Drug related toxicity: All toxicity seen that was considered possibly, probably or almost certainly drug related. In addition it includes all infectious events regardless of presumed causation

Toxicity	Description	Gd 1	Gd 2	Gd 3	Gd 4	Overall
Metabolic	Hypernatremia	2	0	0	0	2
	Hyponatremia	1	0	0	0	1
	Elevated urate	1	0	0	0	1
Constitutional	Fatigue	5	0	1	0	6
	Fever	2	0	0	0	2
Blood	Anaemia	0	8	3	0	11
	Lymphopenia	0	1	8	0	9
	Leukocytopenia	1	3	3	2	9
	Neutropenia	0	1	2	4	7
	Thrombocytopenia	0	2	3	6	11
Bleeding	Haematuria	2	0	0	0	2
	Vaginal bleeding (menstrual)	0	0	1	0	1
	Petechiae/bruising	1	0	0	0	1
	Epistaxis	0	0	1	0	1
Infection	Febrile neutropenia	0	0	2	1	3
	Tunnelled line infection	0	1	4	0	5
	Upper respiratory tract infection	1	0	1	0	2
	Chest infection	0	1	0	1	2
	Urinary tract infection	0	1	0	0	1
	Oral Candida	2	0	0	0	2
Gastro-intestinal	Vomiting	1	0	1	0	2
	Nausea	3	0	0	0	3
	Stomatitis	0	1	0	0	1
Hepatic	Elevated liver enzymes	5	0	0	0	5
Renal	Proteinuria	1	0	0	0	1
Endocrine	Hypothyroidism	0	1	0	0	1
Cardio-vascular	Reduction cardiac function	0	1	0	0	1
	Hypotension on day 1	1	0	0	0	1

2.5.4 Response data

Objective clinical responses according to RECIST (Response Evaluation Criteria in Solid Tumours) were observed in a dose dependent fashion. No responses were seen at 370MBq/m^2 and only one at 740MBq/m^2. Six of 9 patients treated at a single administration of 1200 MBq/m^2 or higher had a response (3 = CR and 3 = PR). Two of the responding patients were receiving concurrent corticosteroids (patient 7 and 14), which remained at the same dose throughout the study. These two patients had previously progressed on this dose of steroid. Neither patient with ATLL received a dose at or above the MTD.

Table 2.12 describes the FDG-PET results from the region-growing programme described by Green et al (251). The mean count per voxel for the volume of interest grown was calculated. The values are expressed as a percentage of the base line value so a reduction in tumour size or volume would give a value less than 100%; an increase in size or volume would give a value greater than 100%.

Green et al defined a partial response as a reduction to less than 85% of baseline uptake; stable disease between 85-100% and progressive disease greater than 110%. A complete response was not defined. Validation was in solid tumours not lymphoma (251). Combining PET and CT was in accordance with the International Harmonization Project criteria (249).

Table 2.12 Response data
Best response with imaging at 4 weeks and confirmed at 8 weeks.

Patient	Administered activity (MbQ/m^2)	FDG-PET (visual analysis)	FDG-PET quantitative assessment (baseline =100)	CT (WHO criteria)	Combined (Cheson criteria)
1	370	Not done	Not done	Not done	Clinical PD
2	370	Not done	Not done	PD	PD (CT alone)
3	370	PR	67 (PR)	SD	SD
4	740, 1480, 2220	CR	0 (CR)	PR	CR
5	740, 1480	CR	0 (CR)	CR	CR
6	740	PD	394 (PD)	SD	PD
7	1480, 2220, 2960	PR	0 (CR)	PR	PR
8	740	Not done	Not done	Not done	Clinical PD
9	740, 1480, 2220	SD	137 (PD)	SD	SD
10	1200	PR	91 (SD)	SD	SD
11	1200, 1200	PR	Not possible	PR	PR
12	1200, 1200	CR	Not possible	SD	CR
13	1480	PD	70 (PR)	PD	PD
14	1480	PR	50 (PR)	PR	PR

* For the experimental region growing program as devised by Green et al (251), this method has not been validated in lymphoma. The summary of response achieved (CR, PR SD and PD) is therefore based on values obtained for solid tumours and may not be applicable to lymphoma. In particular no criteria for determining CR was made in their paper as no patient attained a CR. These descriptions should therefore be treated with caution and are only given to allow comparison with other methods of assessing response.

There are some inconstancies between the 3 methods of assessing response. The Updated International Working Group Criteria (248) was designed to assess masses that remain post treatment. Prior to these criteria solid masses were determined as a CR (unconfirmed) which was converted to CR if they remained unchanged over 1 year. Using the updated criteria a patient is considered in CR if masses fail to take up FDG-PET on PET-CT. The updated criteria have the advantage of correlating well with prognosis (249). On these criteria patient 4 was updated from PR to CR and patient 12 from SD to PR. Conversely in patient 6 the CT scan did not meet RECIST criteria for progressive disease, but the FDG-PET did. In this situation the FDG-PET is considered more indicative of the patients' prognosis and this is recognized in the combined scoring (248). The combination of FDG-PET and CT as detailed in the final column have now become the standard of care in assessing lymphoma response (248).

The semi-automatic region-growing program is consistent with the FDG-PET as assessed visually apart from patients 7, 9, 10 and 13. In patient 7 the semi-automatic program was not able to determine low intensity disease. It had been predicted that this would be a likely challenge (251). In patient 9 and 10 whilst visual assessment reported SD and a PR respectively, the region growing program showed progression and SD respectively. In both these cases the patients went on to progress relatively rapidly post therapy suggesting that the region growing program may have been more accurate at predicting the lack of a long term response. In patient 13 visually progression was seen which was not confirmed by the region-growing program. This discrepancy appeared to relate to disease close to myocardium making the disease difficult to assess by the region-growing method and the results unreliable.

As the semi-automatic region-growing method remains experimental it should be treated with caution. Before it can be used as part of standard methodology it would need to be validated for use in lymphoma. In addition methods will

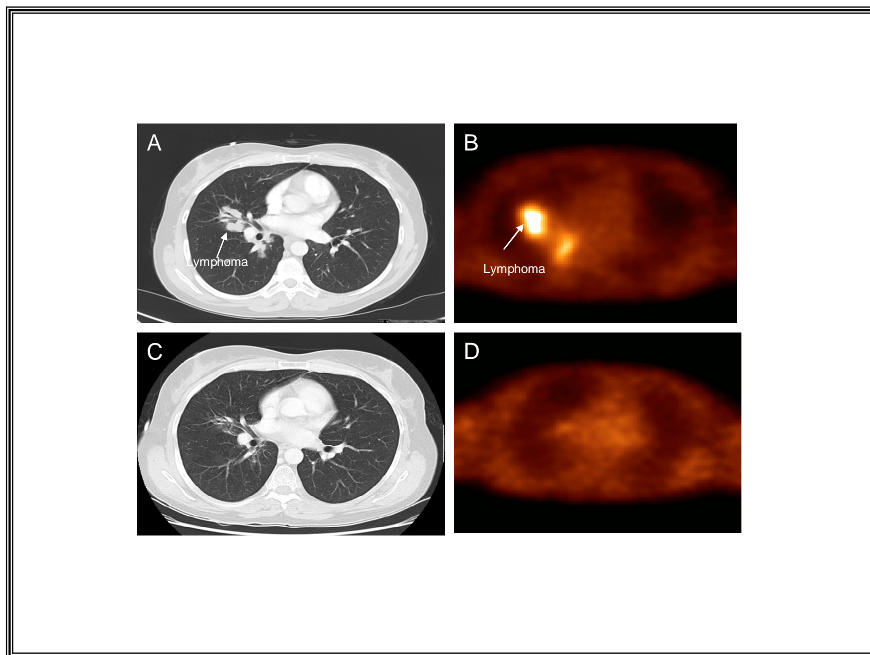
need to be considered to overcome the problems encountered in patients 7 and 13. Until that time the gold standard for lymphoma response assessment will remain the Updated Working Group Criteria as determined by Cheson et al (249).

2.5.4.1 Demonstration of response in 1 patient

Figure 2.3 includes the PET and CT images from patient 14; demonstrating a partial response. The patient subsequently went on to have an allogeneic bone marrow transplant.

Figure 2.3 Response in Patient 14

(A) and (B) pre treatment showing nodal disease (arrowed). (C) and (D) are 4 weeks post treatment.

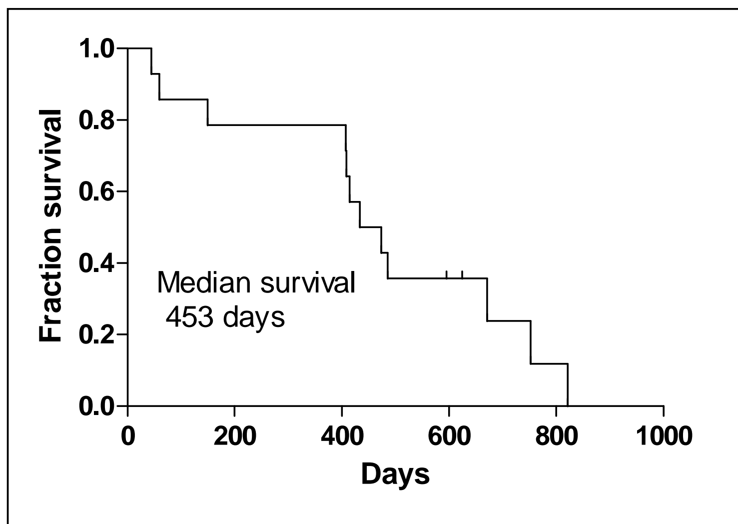


2.5.5 Survival data

The median survival post CHT25 was 453 days (range 44-281) with 2 patients still alive at 624 and 595 days. The data is shown in a Kaplan-Meier plot in Figure 2.4

Figure 2.4 Survival.

Fraction of patients surviving over time (days) using Kaplan-Meier method.



2.5.6 Dosimetry data

Dosimetry data was acquired for 11 of 14 patients. Figure 2.5 shows the biodistribution data, with the decay corrected to collection time, of injected dose of radioactivity, at four time points. It is presented in this way with absolute activities per organ rather than ratios as the absolute values are required to calculate the subsequent dosimetry studies. The data demonstrates retention of radiation in tumour over time compared to normal organs.

The absorbed radiation dose per injected activity for both normal organs and tumour are shown in Figure 2.6 and Figure 2.7. Tumour and normal organ doses are not directly comparable. Normal organ doses include radiation dose from surrounding organs whilst tumour dose does not, tumour dose is therefore underestimated. The low number of patients with tumour dosimetry recorded was due to technical difficulties with the camera calibration making the measurements unreliable. In two patients it was not possible to reliably separate tumour uptake from surrounding tissue. With future studies the acquisition of a new gamma-camera allowing co-registration of SPECT data with CT will alleviate some of these problems encountered in this study. With the caveats above the median muscle to tumour ratio was 1:2 (range from 0.69 to 7.63 times muscle value). The small tumour data set makes these values difficult to interpret.

Figure 2.5 Injected activity % (median)
Mean and ranges of radioactivity uptake by time

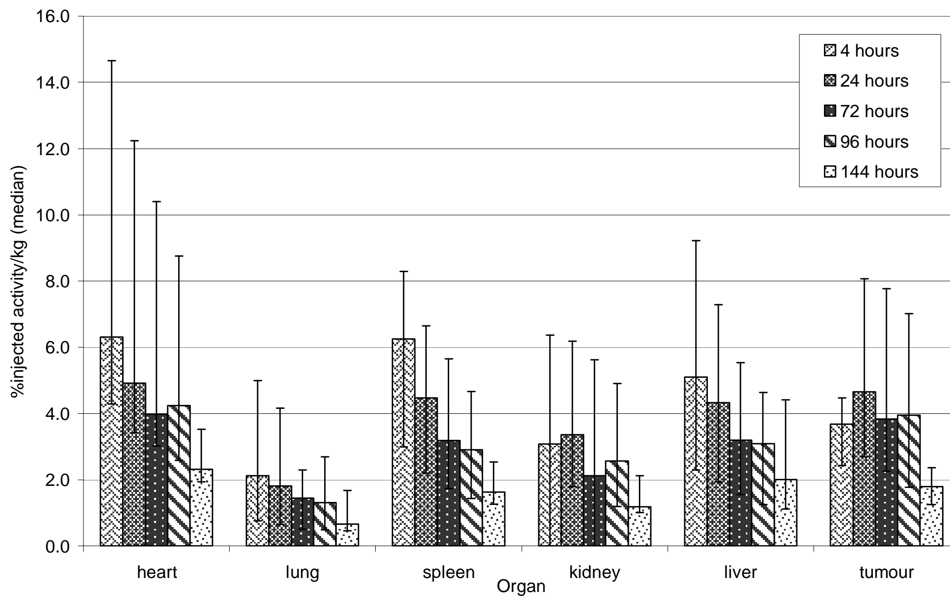


Figure 2.6 Mean and ranges of radiation dose to normal organs.

This includes contribution from other organs

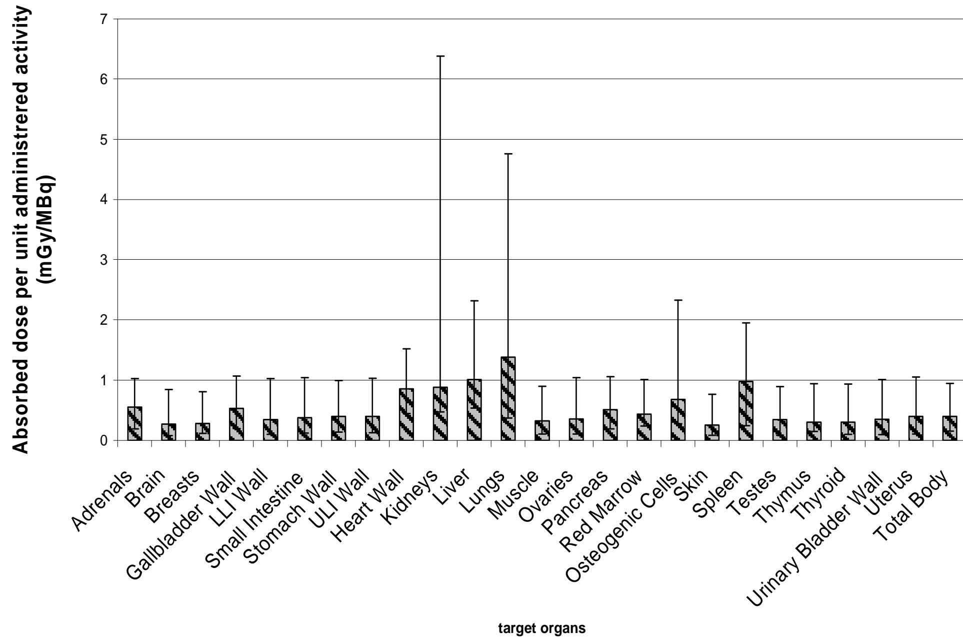


Figure 2.7 Radiation dose to tumour

This excludes contribution from normal organs. The treatment cycle is documented in brackets after the patient number.

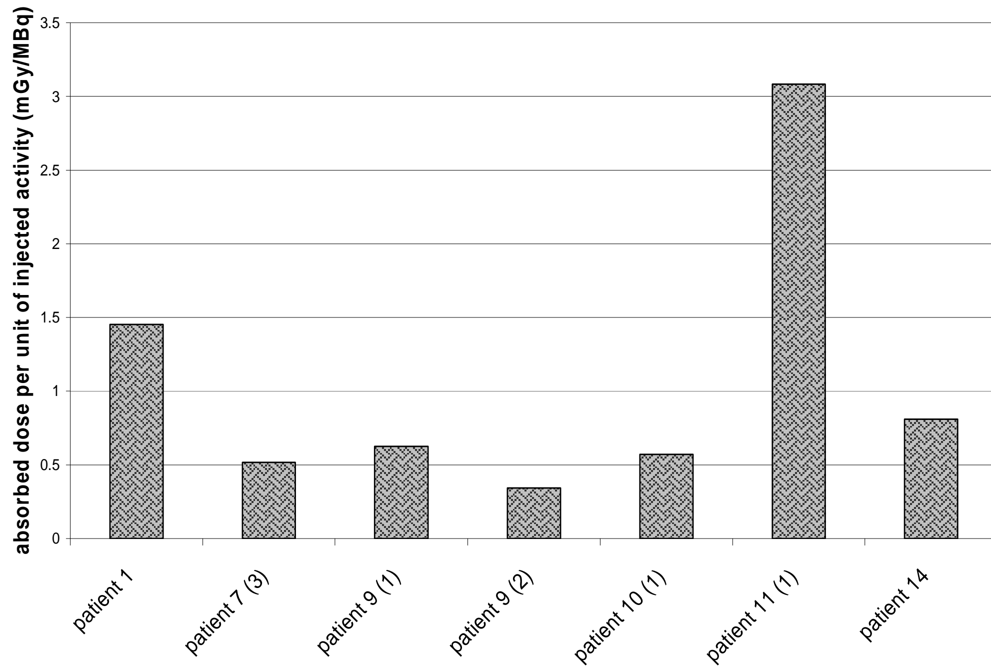


Figure 2.8 and Figure 2.9 consider correlations between absorbed radiation dose and toxicity and response. Figure 2.8 correlates the red marrow absorbed radiation dose to the percentage change in platelet count from start to nadir. Only the first cycle of treatment was considered to avoid ambiguity with the cumulative effects of radiation. Figure 2.9 correlates tumour absorbed radiation dose with response in 7 patients. There is no overlap between the 2 groups (those with response compared to those with stable or progressive disease) but the number of data points are too few to draw any conclusions.

Figure 2.8 Correlation red marrow dose and % change platelet count

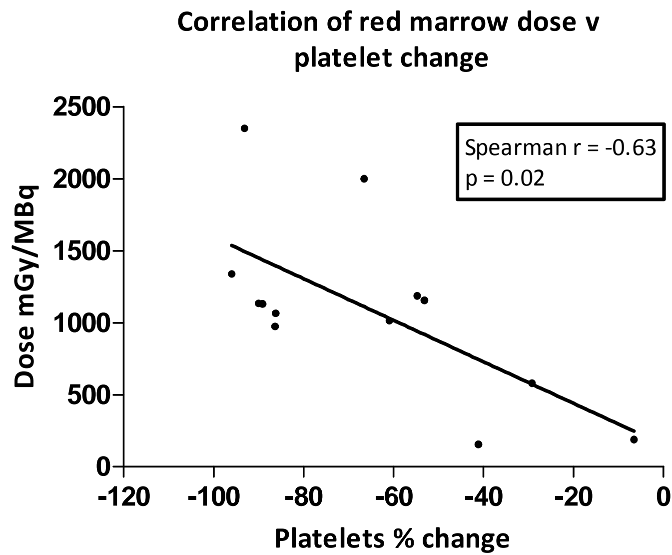
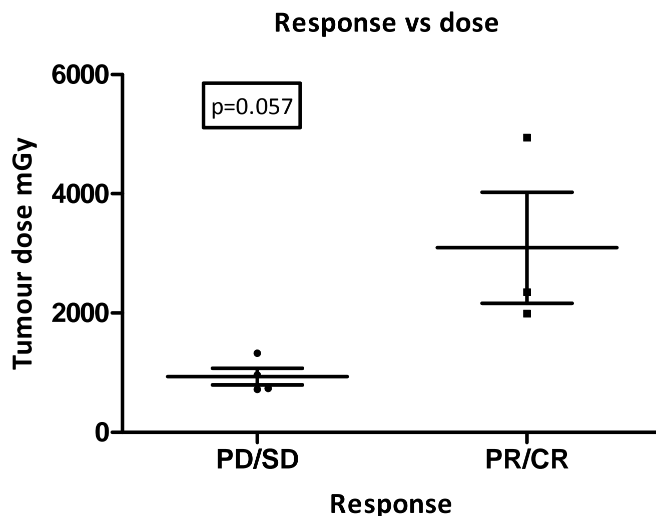


Figure 2.9 Tumour absorbed dose and response (graph and table)
Response was determined using the Updated International Working Group Response Criteria (Cheson Criteria)(249).



Response	Values in mGy			
PD or SD	963	736	723	1325
PR or CR	4945	1991	2350	

2.5.7 Pharmacokinetics

Table 2.9 shows the pharmacokinetics for 22 treatments; 13 can be modelled using a bi-exponential model while 9 fit a mono-exponential. This difference may relate to data collection point positioning masking an initial rapid clearance. Using the mono- or bi-exponential model as appropriate it is possible to calculate the 50% and 90% clearance which was 44 and 193.5 hours respectively. For the 13 treatments possible to fit to a bi-exponential model the t ½ α was 6.1 hours and the t ½ β was 86.7 hours. As predicted the chimeric antibody half-life was longer than the murine anti-CD25 anti-Tac antibody labelled with ^{90}Y (t ½ α of 3.51 hours and a t ½ β of 54 hours).

Table 2.13 Pharmacokinetics A_{0a}, A_{0b}: amplitudes, $T_{½a}$,$T_{½b}$: half lives of the 1st and 2nd components of the bi-exponential curve, %ID/kg: percentage injected dose per kilogram, 50% & 10%: time taken for modelled activity to fall to 50 or 10% of initial value.							
Dose level	**Patient No**	**A0a(% ID/kg)**	**A0b(% ID/kg)**	**T½a- (h)**	**T½b (h)**	**50.0%(h)**	**10.0%(h)**
370	1	0.7	3.6	6.1	98.4	73.5	305.5
370	3.1	1.9	6.5	3.2	58.3	37	174
740	3.2		2.7		8	8	28.8
740	4.1	1.8	8.5	0.4	60.4	43.5	186
740	5.1	6	7.1	10.7	422.5	60*	1025*
740	6	1.3	1.4	0.05	46.3	12.5	120
740	8	3.1	5.5	0.9	57.2	20.5	153
740	9.1		8.2		44.1	44	144
1200	10.1	3.1	5.9	0.9	86.7	33.5	234
1200	11.1		15.5		53	53	176
1200	12.1	7.4	12.7	17.2	113.9	53	300
1200	12.2		9.6		37.5	37	126
1480	4.2	4.5	5.8	1.8	135.6	35	338
1480	5.2	1.9	2.9	16.4	122.7	51	318
1480	7.1	2.9	7	6.3	76	39	214
1480	9.2		8		43.9	44	144
1480	13		5.5		57.8	58	192
1480	14	2.8	4.5	17	108.1	51	285
2220	4.3		3.8		123.4	123	410
2220	7.2		7.9		66.1	66	220
2220	9.3	1.9	3.4	10.3	58.8	30	156
2960	7.3		4.6		58.6	58.5	195
Median						44	193.5

(*) The long decay shown might have been affected by the small number of blood samples

2.5.8 Soluble IL2-R

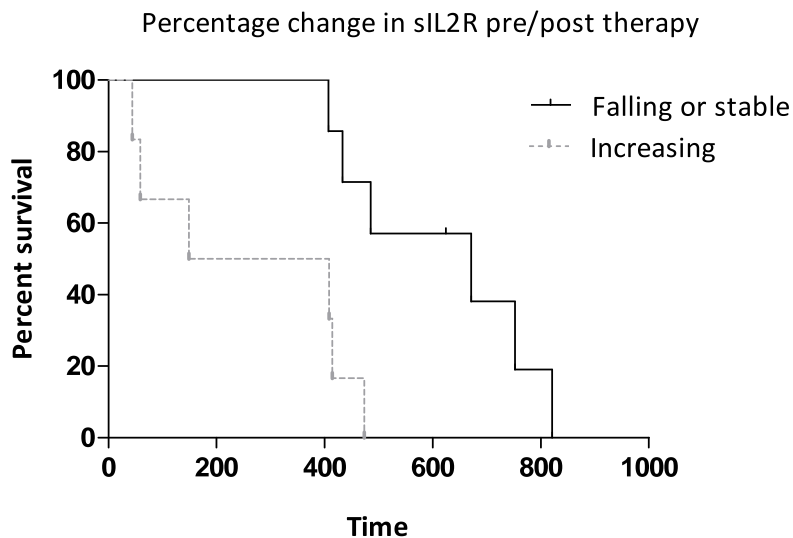
The median pre treatment soluble IL-2R (sIL2-R) was 2803.3 U/ml (range 580-66080 U/ml). This compares with median normal values for the assay of 521U/ml (range 269 – 1116 U/ml). The level of pre-treatment sIL2-R was not found to be a prognostic factor for survival in this small patient sample; patients with above median values had a survival of 407 days compared to 485 days, which was not significantly different (Kaplan-Meier method, Mantel-Cox x^2 0.17,

p = 0.68). Previous work had shown a patient sub-group with a low sIL2-R and a good prognosis (252). Only 1 of our patients fell into this good prognostic category.

The molar ratio between the CHT25 antibody and sIL-2R levels in plasma at the moment post injection (using plasma volume from standard man) was calculated to be a median of 49 to 1 (with a range from 2:1 – 260:1). There was no clear relationship between the ratio and response.

The percent change in sIL-2R values were calculated pre and post therapy. Patients with falling or stable values had a median survival of 671 days compared with 279 in those with a rising value (p=0.0049 Log rank (Mantel-Cox) test. The survival curves are demonstrated in Figure 2.10.

Figure 2.10 Survival according to % change in sIL2R pre and post therapy



2.5.9 Immunogenicity

Three patients received 3 treatments, 4 had 2 treatments and 7 had 1. One patient became HACA positive (patient 1) at 45 days after the 1st treatment. There was rapid blood clearance of CHT-25 in patient 3 without positive HACA and this was not explained. Patient 9 was non-specifically positive in the HACA assay to both irrelevant control antibody and PBS (phosphate buffered solution) at baseline; values fell with therapy suggesting non-specific binding. One patient post-allograft was ineligible for study entry due to a positive HACA possibly representing an immune anti-tumour response.

2.6 Conclusions and Discussion

There is limited experience with chimeric or human antibodies in RIT. This study determines the pharmacokinetic, pharmacodynamic, related toxicity and tumour response, and reports generally favourable findings at the MTD. The patients included had poor prognostic features (Table 2.7) with tumours resistant to multiple lines of therapy and concomitant damage to bone marrow.

Dose-limiting toxicity, as predicted, was myelosuppression which was readily managed at the MTD. The only treatment related death occurred in a patient who received more than twice this dose. The rate of non-neutropenic infection was relatively high (Of 10 patients with infections, 3 were neutropenic associated). It is unclear whether this rate was due to modulation of CD25-positive immune cells by therapy or the pre-existing immunosuppression of this group. No evidence of viral re-activation was seen but given the associated lymphopenia, appropriate microbial prophylaxis may be warranted in future studies. It was confirmed that repeat therapy was feasible after haematological recovery. Further chemotherapy was not precluded in patients whose tumour did not respond. Two patients underwent high dose procedures after receiving

CHT25 (one allogeneic bone marrow transplant and one ASCT) and both had successful engraftment.

Response was assessed using ^{18}FDG-PET and CT consistent with current best practice. Of 9 patients treated at or above the MTD the overall response rate was 67% (3 CR and 3 PR). Two further patients had stable disease while 1 had progression. The 2 patients on stable doses of prednisolone responded having previously progressed whilst taking that steroid dose. As a phase I study the response rate was encouraging. The median survival of 453 days was in part due to sustained remissions, some of which were long-term.

Correlation was seen between red marrow absorbed dose and marrow toxicity. Variation in the extent of bone marrow damage pre-CHT25 was not considered. Baechler et al found the main determinant for haematological toxicity was the duration since the preceding chemotherapy regime (253). Flt3-L (Fms like tyrosine kinase ligand) levels have been reported as a means of addressing the degree of marrow vulnerability (254) and would be worthy of inclusion in future trials.

Dosimetry demonstrated a longer retention of activity in tumour compared with normal tissues. To exploit this, ^{131}I with its physical half life of 8 days was chosen as the therapeutic radionuclide. Tumour responses were only recorded in patients with absorbed doses ≥ 1990 mGy showing the potential for dosimetry studies to identify patients suitable for treatment. Although the radiation appears important for tumour response, additional antibody factors may assist. Recent work has considered a possible anti-cancer effect from the depletion of regulatory T cells (255) or through other immune effects. Evidence of a direct anti-cancer immune mediated antibody effect is currently inconclusive. Of note unlabeled antibody without a RIC showed limited benefit with short lived responses (243).

The pharmacokinetics and dosimetry data reveal the chimeric antibody delivered radiation to normal tissues over a more prolonged time than previously reported in the murine CD25 antibody (^{90}Y-antiTac) (16).

The serum sIL2 receptor was elevated in our study, although to a variable extent between patients. In every patient it was found at a lower concentration than its antibody, CHT25 at the time of its administration. The ratio of antibody to sIL-2R appeared adequate for targeting (by dosimetry) and generated a high tumour response rate. Further improvement may be seen by reducing sIL-2 levels prior to CHT25 administration to prevent antibody diversion away from tumour. The two successfully licensed RIC's both administer unlabeled antibody prior to the RIC to overcome the CD20 'sink' on normal cells. An elevated sIL2-R level is known to be an adverse prognostic factor in Hodgkin's lymphoma (252). Our study could not confirm that pre-treatment sIL-2R levels were a prognostic survival factor although this is not definitive due to the small sample size.

One advantage of arming monoclonal antibodies with ^{131}I is that the mean path length (0.4mm) of the radionuclide allows for the eradication of adjacent antigen-negative tumour cells ('the crossfire effect') (10, 11). The longer path length of ^{90}Y results in a greater proportion of the dose being deposited outside small tumour masses and the shorter half life means the prolonged retention of antibody in tumor cannot be exploited. There is potential for investigation for other radionuclides and combinations of radionuclides.

Previously RIT with ^{131}I has been used in phase I/II clinical trials in HL with polyclonal anti-ferritin or anti-CD30 directed antibodies (232, 237). The overall response rates ranged from 36 to 49 % and the major side-effect, as observed here, was haematological toxicity, most notably thrombocytopenia.

Unlabelled monoclonal antibody therapy directed against CD25 exploits the discordance in IL-2R-alpha expression between tumour cells and normal tissue and has an established history in hematopoietic malignancy (243, 244). CHT25 could be useful in tumours other than HL (226, 227) one patient with a T cell lymphoma had a CR in our study.

CHT25 has a potential application in preparation for autologous and allogeneic transplantation giving the possibility of long term disease control to a proportion of patients with relapsed or refractory disease. Demonstrable chemo-sensitivity and maximal reduction in disease burden prior to transplantation are critical predictors of outcome (230, 256). In this study we induced responses and transplanted patients previously considered inappropriate for this treatment. This justifies further investigation of CHT25 either to render patients eligible for transplant or to improve transplant outcome when combined with standard conditioning regimens. It may have a place in the early treatment of poor prognosis patients and in other CD25-positive lymphomas.

In summary, the CHT25 study demonstrates that RIT with a chimeric antibody can be safely administered at a dose of 1200MBq/m^2 giving objective responses in a high proportion of patients with lymphomas expressing CD25. Pharmacokinetic and pharmacodynamic parameters relevant to producing this effect have been shown. CHT25 appears to have potential as a new treatment in this patient group.

Chapter Three: RIT of CEA-expressing Malignancy with ^{131}I-A5B7 and CA4P

Our hypothesis stated that it would be unlikely for single agent RIT to be effective in solid tumours, particularly in bulky disease. Instead we decided to investigate the combination of a Vascular Disrupting Agent (VDA) with RIT. We hoped to demonstrate synergy between the two agents, due to their differing modes and sites of action within the tumour. Scheduling of the agents was designed to facilitate RIC trapping in tumour. Synergy had already been seen in animal experiments between the two compounds with transformation of a sub-curative therapy to a curative one.

The VDA that was chosen was Combretastatin-4-Phosphate (CA4P), which acts by binding tubulin. Initially in this chapter I shall discuss this compound before considering the chosen antibody, A5B7, a murine anti-CEA antibody.

3.1 Combretastatin-A4-phosphate (CA4P)

CA4P is a water-soluble pro-drug that is dephosphorylated into CA4, the active form, before binding rapidly to β-tubulin causing depolymerisation and inhibition of tubulin assembly (257, 258). Newly formed endothelial cells become distorted, leading to blood vessel occlusion and a reduced flow. Mature cells are relatively unaffected as their actin cytoskeleton is more robust (259). CA4P also disrupts endothelial cell signalling via the vascular endothelial cadherin pathway (260).

3.1.1 Pre-clinical studies

CA4P leads to rapid reductions in tumour blood flow in mice with spontaneous tumours, and xenografts (261, 262). The first effects are visible at 10-20 minutes with endothelial cell distortion *in vitro*. Vascular collapse follows at 1 hour leading to a core of haemorrhagic necrosis (263). At 6 hours tumour blood flow is reduced 100 fold with only minimal changes in normal organ flow (262). Tumour blood flow returns to normal by 24 hours post infusion (264) and massive tumour necrosis is visible with 90-99% of tumour cells killed (using 100mg/kg). The effect on tumour growth is minimal as repopulation occurs from the viable rim of peripheral tumour cells supplied by normal blood vessels (263). In our combination study we hypothesize these will be killed by RIT.

3.1.2 Clinical studies

Three Phase I studies have investigated CA4P as a single agent in refractory solid tumours (265-267) using DCE-MRI (Dynamic contrast enhanced – magnetic resonance imaging) to assess blood flow (See Table 3.1 and Table 3.2). Each study used a different dosing schedule; a single dose every 21 days (266), 5 consecutive doses in 21 days (267) and weekly (265). All 3 studies had a reduction in tumour blood flow with little effect on systemic flow. In 2 of the 3 studies, 1 patient had an objective response. Disease stability or minor responses were more common and ranged from 9% (265) to 38% (266).

CA4P was generally well tolerated. The commonest side effects were nausea, headache, tumour pain and tiredness. Most toxicity resolved within 2 days. Dose limiting toxicities developed between 52mg/m^2 and 114mg/m^2 and are described in Table 3.2. The MTD was defined as between 60-68mg/m^2.

Table 3.1 Phase One studies of CA4P				
Trial	**Number**	**Patient group**	**Monotherapy**	**Response**
Dowlati et al 2002 (183)	25	Refractory solid tumours	Single agent CA4P	1 CR* 11 SD
Rustin et al 2003 (181)	34	Refractory solid tumours	Single agent CA4P	4 SD
Stevenson et al 2003 (182)	37	Refractory solid tumours	Single agent CA4P	1 PR** 16 SD

* A patient with anaplastic thyroid cancer who has remained in complete remission for > 5 years.

** A patient with metastatic soft tissue sarcoma

Table 3.2 Dose-limiting toxicity with CA4P					
	Dose-limiting toxicity encountered (number patients in brackets)				
Dose (mg/m^2)		60	75	90	114
Dowlati et al (183)		Myocardial ischaemia (1) Prolonged QTc (1)		Cardiac ischaemia and dyspnoea (2)	
Rustin et al (181)	Small bowel ischaemia (1)*			Ataxia (1) Dyspnoea, vasovagal and tumour pain (1)	Ataxia (2)
Stevenson et al (182)			Tumour pain (2)		

* Patient died at day 15 from an ischaemic and /or perforated bowel associated with a tumour mass in a previous radiotherapy field. It was defined as treatment related due to possible impairment of mesenteric blood supply to an area already compromised by the radiation.

3.1.3 Combining a VDA with RIT

Pre-clinical studies of the combination suggest that with optimal timing, tumour blood vessel collapse may trap radiolabeled antibody within tumour and increase tumour absorbed dose (123, 268). The optimal schedule for the CA4P in animal models was 24-48 hours after the radioimmunoconjugate (RIC) (268) giving a 90% increase in radiation retention compared to the control of RIT alone (123).

In pre-clinical animal models the combination transformed a sub-curative therapy into a curative one (with both CA4P (123) and DMXAA (269)), although with a narrow therapeutic window. Pedley et al administered CA4P and ^{131}I-A5B7 to nude mice with the colorectal xenograft SW1222. Single agent CA4P had no effect on survival, the RIC prolonged survival by 35 days, but the combination led to cures in 85% of mice (123) with no viable tumour cells found at the implantation site.

3.2 A5B7 Antibody

The A5B7 Antibody is a murine monoclonal IgG antibody targeting CEA. In normal colonic epithelium it reacts against the cellular apical regions. In colonic carcinoma it reacts against both the basal and luminal cellular surface at moderate or intense levels. The targeting of the basal region is important as this areas is more accessible to antibody (270). Whilst the exact epitope that A5B7 reacts against remains unknown it is specific to CEA without cross-reactivity to liver, erythrocytes or neutrophils (271).

A7B7 has been demonstrated to localise to human colon carcinoma xenografts by Pedley et al (123, 272). A previous single-agent phase I study of ^{131}I-A5B7 found an MTD of 2400MBq/m^2 with bone marrow toxicity dose-limiting (94).

The half-life of ^{131}I-A5B7 was 28.6 hours in blood and 59.5 hours in tumour. The single-agent response rate was 10%. The MTD of CA4P from single-agent studies was between 60-68mg/m^2 (265, 266) with a mean plasma half-life of 0.2 hours, 2 hours for CA4 and a further 4.3 hours for the subsequent product CA4G.

3.3 Methods

The A5B7 CA4P study was a two-centre, open-label, non-randomized dose-escalation study designed to evaluate the combination of RIT, ^{131}I-A5B7, and CA4P. The patients were recruited from the Royal Free Hospital NHS Trust and Mount Vernon Hospital.

3.3.1 Trial Objectives

The primary objectives were to determine the safety profile of the combination of ^{131}I-A5B7 and CA4P and to determine the relationship between efficacy and tumour blood flow reduction by CA4P.

Secondary objectives were to determine the MTD of the combination and to assess pharmacokinetics and pharmacodynamics.

3.3.2 Pharmaceutical

A5B7 was produced at the Biotherapeutics Development Unit (Cancer Research UK, Clare Hall Laboratories) by purification from tissue culture fluid using protein A chromatography and ion exchange chromatography. It was then formulated in isotonic phosphate buffered solution (PBS) and stored at 4°C. It was radiolabeled with ^{131}I at the Royal Free Hospital using the N-bromosuccinamide method (247), known to have a labelling efficiency of 88-

94% without loss of immunoreactivity (94). ^{131}I was obtained from Amersham Pharmacia Biotech as sodium iodide. After labelling drug stability was estimated at 4 hours.

CA4P was supplied by Oxigene Inc., to the Cancer Research UK Formulation Unit who performed the final labelling operations. It was supplied as a sterile freeze-dried, disodium salt in amber vials. It is light sensitive and was stored refrigerated in the dark.

3.3.3 Selection of starting dose and schedule

The starting doses of both agents were lower than their respective MTDs from their single agent studies for safety. For CA4P, 45 (50)* mg/m^2 was considered to be the lowest dose with a reasonable chance of reducing tumour blood flow with a planned escalation to 55 (60.5)* mg/m^2, the MTD recommended by Oxigene. The starting dose of ^{131}I was 1800 MBq/m^2, which is 25% below its MTD from single agent studies. It was planned to increase to the single-agent MTD of 2400 MBq/m^2. No additive toxicity was anticipated from previous animal studies (75, 185) as the two compounds had differing mechanisms of action and toxicity profiles. The A5B7 antibody was given at a fixed dose of 10mg.

* The CA4P dose is presented as the free acid form concentration and is 90% of the dose representing the disodium salt. The equivalent salt form concentration is shown in brackets.

A traditional 3 + 3 dose-escalation strategy was employed. Each dose was administered in cohorts of 3 patients. If a DLT occurred the cohort was expanded to 6 patients. Dose escalation was allowed providing 0 of 3 patients or less than 2 of 6 patients experienced a DLT. A minimum time interval of 7 days was required before treating a new patient in the same cohort, and 4 weeks if escalating to a new cohort to allow time to assess for toxicity.

Table 3.3 Planned dose escalation schedule			
Planned cohort	**CA4P* (mg/m^2)**	**A5B7 (mg)**	**131-Iodine (MBq/m^2)**
1	45 (50)	10	1800
2	55 (60.5)	10	1800
3	55 (60.5)	10	2100
4	55 (60.5)	10	2400
* The CA4P dose is presented as the free acid form concentration and is 90% of the dose representing the disodium salt. The equivalent salt form concentration is shown in brackets.			

If there was no evidence of disease progression on day 57 and the eligibility criteria were still met a patient could be retreated at the same dose. If HAMA positivity had occurred, CA4P could be given alone. The initial planned study size was 14 patients. If no evidence of efficacy was seen at that point the trial would be terminated. If responses were observed a further 11 patients would be recruited.

3.3.4 Agent administration

Radiation protection issues meant the DCE-MRI could not be performed after administration of the RIC. To assess the vascular response to CA4P it was decided to administer CA4P 1-2 weeks prior to the RIC with DCE-MRI assessment pre and post and pharmacokinetic assessment. Previous work had demonstrated recovery of tumour vasculature 24 hours after CA4P (263) so this protocol was considered unlikely to reduce subsequent tumour ^{131}I-A5B7 uptake. ^{131}I-A5B7 was given on day 0 with CA4P on days 3, 4 and weekly thereafter for up to seven weeks or until unacceptable toxicity or tumour progression occurred. The rationale for CA4P on day 3 was based on animal

work suggesting this is the optimal schedule to trap ^{131}I-A5B7 in tumour (123). Day 4 was added to maximise this effect.

CA4P was reconstituted with 11ml sterile water then diluted with 100-150ml 0.9% saline. It could then be kept for up to 4 hours if protected from light. A line filter removed particulates during the 10 minute infusion time. Patients were monitored for 4-6 hours afterwards for cardiovascular instability.

^{131}I-A5B7 was given intravenously over 30-40 minutes in 50ml 0.9% saline using a shielded syringe driver. Radiation protection personnel were present to ensure compliance with the Ionization Radiation Regulations. Resuscitation facilities were available.

3.3.5 Inclusion and Exclusion criteria

3.3.5.1 Inclusion Criteria

- Histological diagnosis of a CEA-expressing gastrointestinal malignancy. Plasma CEA level 10-1000 μg/L or CEA-expression on tumour blocks
- Measurable disease clinically or on CT / MRI assessable by RECIST criteria.
- Completed or not eligible for standard anti-cancer therapies.
- Disease suitable for blood flow evaluation by DCE-MRI (> 2cm / not in sites that move with respiration or vascular pulsation). Be able to tolerate MRI.
- A normal ECG (Electrocardiography) with a QTc interval < 450msec
- Age > 18 year
- Life expectancy > 3 months
- Performance status (WHO criteria) 0 or 1

- Adequate organ function for the study including:
  - Neutrophil count > 1.5 x 10^9/L
  - Haemoglobin > 9g/dl (to transfuse to > 10g/dl)
  - Platelet count > 100 x 10^9/L
  - Calculated creatinine clearance (uncorrected) > 50ml/min
  - Plasma bilirubin, ALT and AST < 2 x the upper limit of normal (ULN). (5 x ULN allowed for transaminases in the context of liver metastasis.
  - INR < 1.1 (or if on 1mg warfarin < 1.5)
  - Potassium and magnesium within normal ranges
- All > grade 1 toxicity (except alopecia) from prior therapy resolved.
- Capable of giving written informed consent

3.3.5.2 Exclusion Criteria

- At risk of cardiac complications: a prior history of angina, myocardial infarction, congestive heart failure, non-controlled atrial arrhythmias or clinically significant arrhythmias, uncontrolled hypertension (> 150/100 irrespective of medication) or an abnormal ECG.
- Medication that prolonged the QTc interval, anti-coagulants (excepting 1mg warfarin for tunnelled central lines) or naproxen.
- Pregnancy, breast feeding or inability to use contraception.
- Previous curative doses of radiotherapy to thorax or abdomen at any time or post-operatively to the pelvis due to risk of perforation. Pre-operative radiotherapy to rectum allowed if that section of bowel then removed.
- Serious non-malignant systemic disease: uncontrolled infection, hepatitis B and C, HIV, auto-immune disorders, inflammatory bowel disease or moderate to severe peripheral neuropathy.

- Major surgery in the preceding 3-4 weeks or anti-cancer therapy in the past 4 weeks (excluding palliative doses of radiotherapy to non-target lesions).
- Presence of brain metastases
- Any additional active malignancy excluding adequately treated in situ carcinoma of the cervix, uteri, basal or squamous cell cancer of the skin.
- Positive HAMA or an allergy to iodine or mouse proteins.

3.3.6 Pre-trial investigations

All assessments were performed within in the time-scales in Table 3.4.

Table 3.4 Pre-trial investigations

Date pre-therapy	**Nature of investigation**
Within 12 weeks	Blood taken for HAMA
Within 4 weeks (preferably within 2)	Written informed consent CT and if required MRI for disease assessment FDG-PET and blood glucose
Within 2 weeks	Tumour markers in serum (CEA and CA19-9 (carbohydrate antigen), thyroid function (TSH and free T4 (thyroxine)
Within 1 week	Medical history (symptoms, vital signs, urinalysis, body surface area, performance status and physical examination) Assessment of drug history Basic investigations; ECG, CXR (Chest X-ray), creatinine clearance. Blood tests (haematology*, biochemistry** and clotting***). Register with Cancer Research UK Drug Development Office

Haematology: haemoglobin, white cell count, lymphocytes, neutrophils, and platelet count.

** Biochemistry: electrolytes, urea, creatinine, urate, total protein, albumin, bilirubin, AST, ALT, ALP (alkaline phosphatase), GGT, glucose and magnesium

*** Clotting includes PT, INR and APPT

3.3.7 Trial follow-up

Patients were followed up for a minimum of 8 weeks or until trial-drug related toxicity had resolved (see Table 3.5). Potassium iodide was administered from

day -2 until day 11 (50-60mg three times a day) to protect the thyroid gland from radioactive iodine uptake. DCE-MRI was performed at 48 and 24 hours prior to initial CA4P and 4 hours after. Pharmacokinetic data was collected for CA4P on its initial administration and day 1 after ^{131}I-A5B7 infusion. Patients remained as in-patients until determined safe for discharge by the radiation protection team. Whilst they remained in-patients daily SPECT scans were performed.

Table 3.5 Follow-up protocol

Protocol for follow-up post discharge	**Nature of investigation**
Weekly	Clinical assessment (examination, vital signs, physical examination and performance status) Basic investigations including ECG, Blood investigations (haematology and biochemistry)
Day 32 and day 53	Tumour markers, thyroid function, HAMA estimation
Day 29 and day 57	Disease response assessment by CT and MRI if required
Day 18, 29 and 57	Disease response by FDG-PET

3.3.8 Toxicity

Toxicity was graded using the National Cancer Institute Common toxicity criteria (CTC) (version 2). The highest grade in any one particular cycle was recorded.

3.3.8.1 Definition of a Dose-limiting toxicity

A DLT was defined as a toxic event, almost certainly or probably drug related. Any grade 3 or 4 toxicity was defined as dose-limiting, excepting hair loss, grade 3 nausea, grade 3 or 4 vomiting or diarrhoea in patients not adequately supported. Additional DLTs specific to CA4P were cardiac (detailed below) and grade 2-4 neurotoxicity. Any death thought to be drug-related was also a DLT.

Haematological dose limiting toxicities

- Grade 4 neutropenia (< 0.5 x 10^9/l) for > 5 days
- Grade 3 or 4 febrile neutropenia; fever > 38.5°C and a neutrophil count < 1.0 x 10^9/l
- Grade 3 or 4 thrombocytopenia (platelet count < 50 x 10^9/L)
- Grade 3 or 4 anaemia (Haemoglobin < 8.0 g/dl)

Cardiac dose limiting toxicities (specific for CA4P / not necessarily CTC criteria)

- QTc prolongation of > 500 msec on ECG
- Grade 2, 3 or 4 ventricular arrhythmia; second or third degree AV block, severe sinus bradycardia (< 45 bpm) or tachycardia (>120 bpm), persistent supraventricular arrhythmia (e.g. atrial fibrillation or flutter) lasting for > 24 hours, ventricular tachycardia (> 9 concurrent beats / any length of torsades de pointes) or unexplained recurrent syncope.

3.3.8.2 Definition of maximum tolerated dose (MTD)

The MTD was the dose at or below that where DLT's were found to occur. Toxicity was considered manageable at this level, acceptable and generally reversible.

3.3.9 Response Assessment

All patients who received 1 cycle were assessed for response with CT at 4 and 8 weeks post therapy (RECIST criteria). FDG-PET was performed in selected patients' pre-therapy, at 2-4 weeks and 8 weeks post therapy. An ADAC Vertex

Plus Dual Head Co-incidence camera hybrid SPECT/PET camera (Phillips-ADAC, Eindhoven, Netherlands) was used.

Analysis was with an iterative threshold based region growing program (251). The methodology for image acquisition and analysis were described in Chapter 2. A partial response was defined as a 15% or greater reduction from baseline; progressive disease was an increase of ≥ 10% and stable disease between those two limits. These ranges have been validated in solid tumours (251).

3.5.10 SPECT and dosimetry data

SPECT scans were performed at 4, 24, 48 and 72 hours post administration of ^{131}I-A5B7 with the hybrid SPECT/PET camera. ROI based analysis was performed on both tumour and normal tissues and used to calculate dosimetry as previously described in Chapter 2.

3.5.11 Vascular parameters with DCE-MRI

All patients had DCE-MRI to assess tumour blood flow. Gadolinium diethylenetriampentaacetate (Gd-DTPA) (Magnevist) was used as a contrast agent whilst a dynamic series of T1 weighted images were acquired. Gd-DTPA is a paramagnetic agent that enhances MRI signal intensity in proportion to its concentration. The rate of leakage of Gd-DTPA into the extracellular space can be described with a compartmental model (273). In the leaky, tumour-associated blood vessels Gd-TPA diffuses more easily and quickly into the tumour extracellular space. Rapid uptake with high levels of enhancement over time is consistent with perfused tumour (125).

Results can be quantified using two methods; K^{trans} and initial area under the Gd-DTPA contrast agent time curve (IAUGC) (274). K^{trans} is the volume transfer constant between blood plasma and the tumour extracellular space. It reflects both tumour blood flow and permeability. In a rat model CA4P led to a statistically significant fall in K^{trans} at 1 and 6 hours post administration, compared to baseline. No difference was seen in a control group injected with saline (275).

Rustin et al considered DCE-MRI in 21 patients treated with CA4P and demonstrated reproducibility between 2 scans prior to therapy and a significant mean reduction in K^{trans} at 4 and 24 hours in patients receiving at least 46.8 (52) mg/m^2 or greater . No significant reductions were seen in muscle K^{trans} (274).

Based on these studies (265, 274) patients had two MRI scans prior to study entry, to derive a baseline value and assess intra-patient variability. DCE-MRI was then repeated 4 hours post using a 1.5T Siemens Symphony scanner.

3.5.12 Pharmacokinetics

Pharmacokinetics for the CA4P administration, were taken with the initial administration of CA4P 1-2 weeks prior to the administration of the ^{131}I-A5B7. Due to radiation safety concerns no further pharmacokinetics were taken for the CA4P administrations on Days 3 and 4 post ^{131}I-A5B7 to exclude any interaction with the RIC. No interaction was expected to occur with the VDA pharmacokinetics.

Peripheral blood was taken pre-initial administration of CA4P and then at 5, 30 and 45 minutes and 1, 2 and 4 hours. It was kept at 4°C and centrifuged within 30 minutes (3000 rpm for 10-15 minutes). The plasma was stored at -70°C. The analysis used non-compartmental models to produce the maximum observed

plasma concentration (Cmax), time to reach Cmax (Tmax) and the area under the plasma concentration curve (AUC) and terminal elimination half-life (T/half). The same values were calculated for CA4.

Blood (1ml) was taken pre-administration of ^{131}I-A5B7 and at 5 minutes, 4, 24, 48, 72 and 96 hours post infusion and stored in the radioactive storage area until safe to analyze. A Packard Cobra 11th series Auto gamma counter was used to measure ^{131}I activity and decay correction applied to calculate the % injected dose/kg blood at each time point.

3.5.13 Immunogenicity

HAMA was tested pre-study entry and on days 32 and 53. Peripheral blood was centrifuged at 3000rpm for 10-15 minutes and serum separated into a second tube. It was divided into 2 aliquots with 1 analyzed immediately using a standard ELISA protocol against positive and negative controls and the other stored at -70°C.

3.6 Results

3.6.1 Patient Characteristics

Between January 2004 and April 2007 88 patients were screened and 12 patients were recruited. The commonest reason for not entering a patient was their unsuitability for assessment by DCE-MRI (19 patients), patient refusal (11 patients), a negative CEA or a serum CEA value > 1000 (9 patients) or a WHO score > 1 (9 patients). Of those patients recruited 11, had primary colorectal cancer and 1 primary pancreatic cancer. Other characteristics are detailed in Table 3.6.

Table 3.6 Patient Characteristics	
Patient Characteristics	**No patients**
Number of patients treated	12
Sex	
Male	7
Female	5
Age	
Median (range) in years	64 (32-77)
Primary Tumour site	
Colorectal	11
Pancreas	1
Performance Status (WHO)	
0	6
1	6
Number of patients previously treated with	
Biological therapy	1
Radiotherapy	3
Chemotherapy	12
Surgery	12
Number of patients receiving 'x' prior regimens of chemotherapy	
1	3
2	6
3	2
4	0
5	1

3.6.2 Administered activity received

Although the starting doses of both agents were lower than the MTD defined by single-agent studies, two dose-limiting events occurred in the first cohort. Both were myelosuppression and attributed to the RIT component. Consequently the dose of RIT was de-escalated but the dose of CA4P increased to levels that previously had been demonstrated to reduce tumour blood flow. In this second cohort a further 3 episodes of dose-limiting toxicity occurred and the study was terminated. A full summary of doses and injected activities administered are in Table 3.7.

<table>
<tr><th colspan="7">Table 3.7 Injected activities / doses administered</th></tr>
<tr><th>Patient Number</th><th>Dose Cohort</th><th>131-I-A5B7 (MBq/m2)</th><th>CA4P (mg/m2)</th><th>No 131I-A5B7 infusions</th><th>No CA4P infusions</th><th>No DLT</th></tr>
<tr><td>1</td><td rowspan="6">1</td><td rowspan="6">1800</td><td rowspan="6">45</td><td>1</td><td>4</td><td>DLT</td></tr>
<tr><td>2</td><td>1</td><td>5</td><td></td></tr>
<tr><td>3</td><td>1</td><td>4</td><td></td></tr>
<tr><td>4</td><td>2</td><td>16</td><td></td></tr>
<tr><td>5</td><td>1</td><td>6</td><td></td></tr>
<tr><td>6</td><td>1</td><td>10</td><td>DLT</td></tr>
<tr><td>7</td><td rowspan="6">2</td><td rowspan="3">1800</td><td rowspan="6">54</td><td>1</td><td>3</td><td>DLT</td></tr>
<tr><td>8</td><td>1</td><td>8</td><td>DLT</td></tr>
<tr><td>9</td><td>1</td><td>3</td><td></td></tr>
<tr><td>10</td><td>Not given</td><td>0</td><td>1</td><td></td></tr>
<tr><td>11</td><td rowspan="2">1600</td><td>1</td><td>5</td><td></td></tr>
<tr><td>12</td><td>1</td><td>7</td><td>DLT</td></tr>
</table>

3.6.3 Toxicity

All adverse events are described in Table 3.8 with episodes of grade 3 and 4 toxicity given in greater detail in Table 3.9. In the first cohort 2 patients developed grade 4 neutropenia for > 5 days (20 and 7 days respectively). Neither patient became septic although one had cellulitis around the site of a previous paracentesis.

Table 3.8 Summary of all toxicities encountered Toxicity occurring in 4 or more patients is highlighted in bold			
	Number of Patients (n=12)		
	All toxicity determined to be drug related		
	A5B7 related	CA4P related	Attributed both
Allergic reaction	**2**	**2**	
Bone marrow			
Anaemia	7	**0**	**1**
Lymphopenia	7	**2**	**1**
Neutropenia	9	**0**	**1**
Thrombocytopenia	10	**0**	**0**
Cardiovascular			
Sinus Bradycardia	**0**	5	**0**
Sinus Tachycardia	**3**	5	**0**
Cardiac Ischaemia	**0**	**2**	**0**
Prolonged QTc interval	**0**	**1**	**0**
Hypertension	5	7	**0**
Hypotension	**0**	4	**0**
Coagulation			
Prolonged APTT[a]	**0**	**0**	**2**
Prolonged PT[b]	**0**	**0**	**2**
Constitutional			
Fatigue	**0**	**2**	**1**
Fever	5	6	**0**
Rigors / Chills	**3**	**1**	**0**
Endocrine			
Low TSH[c]	**1**	**0**	**0**
Hypothyroidism	**1**	**0**	**0**
Gastrointestinal			
Anorexia	**0**	**1**	**0**
Constipation	**0**	**0**	**1**
Diarrhoea	**1**	**1**	**0**
Nausea	**1**	5	**1**
Stomatitis	**0**	**1**	**1**
Vomiting	**0**	**2**	**1**

Hepatic			
Raised ALT[d]	**0**	**1**	**0**
Raised Alkaline Phosphatase	**0**	**1**	**0**
Raised GGT[e]	**0**	**1**	**0**
Hypoalbuminaemia	**0**	**1**	**0**
Neurological			
Ataxia	**0**	**1**	**0**
Pain			
Tumour Pain	**0**	4	**0**
Non-tumour Pain	**0**	5	**1**
Renal / Genitourinary			
Urinary Frequency / Urgency	**0**	**2**	**0**

[a] Activated Partial Thromboplastin time, [b] Prothrombin, [c] Thyroid Stimulating hormone

[d] Alanine Aminotransferase, [e] Gamma glutamyl transpeptidase

Table 3.9 Grade 3 or 4 toxicities

Patient no	DLT	Onset	Duration	Comment
1	Grade 4 neutropenia > 5 days	Day 51	1 month	Cellulitis at paracentesis site
6	Grade 4 neutropenia > 5 days	Day 31	7 days	Remained well
7	Grade 3 ataxia	Day 1st CA4P	3 days	Steroid with subsequent CA4P
8	Grade 4 neutropenia > 5 days	Day 31	7 days	Remained well
12	Grade 4 neutropenia > 5 days	Day 30 and day 39	8 and 6 days respectively	Remained well

In the second cohort 2 patients developed grade 4 neutropenia for > 5 days (7 days, then 8 and 6 days in the same patient). There was 1 episode of grade 3 ataxia occurring within 24 hours of CA4P and continuing for 3 days. Subsequent infusions for that patient were pre-medicated with dexamethasone.

Cardiovascular toxicity included hypertension, hypotension, sinus bradycardia and tachycardia. Hypertension tended to be mild and self-limiting and only 1 patient required therapy due to associated grade 2 cardiac ischaemia. Tumour site pain post CA4P occurred in 4 patients with a median time to onset of 72 minutes (range 45-177 minutes). Median duration was 60 minutes (5-240 minutes). Mild nausea was common and occurred in 5 patients post CA4P administration. Two patients had mild allergic reactions to ^{131}I-A5B7.

3.6.4 Response Data

Ten patients were evaluable for response on days 29 and 57. Seven patients had early progression by day 29; two had stable disease at day 29 but progressed by day 57. Patient 4 had stable disease at day 57 and received a second cycle of ^{131}I-A5B7 prior to subsequent progression. Patient 6 had a minor response in an iliac lymph node with a fall in CA19-9 from 10507 on day 1 to 2033 IU/ml.

As part of response assessment 4 patients had ^{18}F-FDG-PET performed. Response assessment analysis was performed by visual analysis (blinded to patient identity and scan order) and by semi-quantitative analysis (Table 3.10) (251). All 4 patients assessed with FDG-PET had progressive disease at 4 and then 8 weeks. There was good correlation between FDG-PET and CT.

Table 3.10 Response assessment using FDG-PET semi-quantitative analysis The semi-quantitative analysis assumes the baseline value is 100 and all the subsequent scans are compared to that baseline value. Due to clinical progression there was no day 57 scan on patient 11.			
Patient No	**Pre treatment**	**Day 29 scan**	**Day 57 scan**
2	100	128	237
3	100	192	243
9	100	393	1002
11	100	214	

3.6.5 Dosimetry Data

Nine patients had serial SPECT imaging performed for dosimetry. Patient 3 had no 4 hour scan, patient 10 did not receive ^{131}I-A5B7 and patient 12 only had 1 scan performed. Patient 4 had two cycles of ^{131}I-A5B7 and data was collected for both treatments.

Figure 3.1 shows the percentage of injected dose per kilogram in both normal organs and tumour. It demonstrates evidence of retention in tumour over time in contrast to normal organs. Figure 3.2 and Figure 3.3 show the median absorbed radiation doses to normal organs and tumour respectively. The two graphs are not directly comparable, as the tumour-absorbed doses do not incorporate radiation doses from surrounding organs due to the limitations of the dosimetry program and the lack of co-registered SPECT and anatomical imaging. The absorbed dose to tumour is a relative under-estimation. In patients 5, 6, 8 and 9 radiation doses in tumour are higher than that found in normal organs (excluding lungs).

Figure 3.1 Normal organ and tumour % injected activity / kg

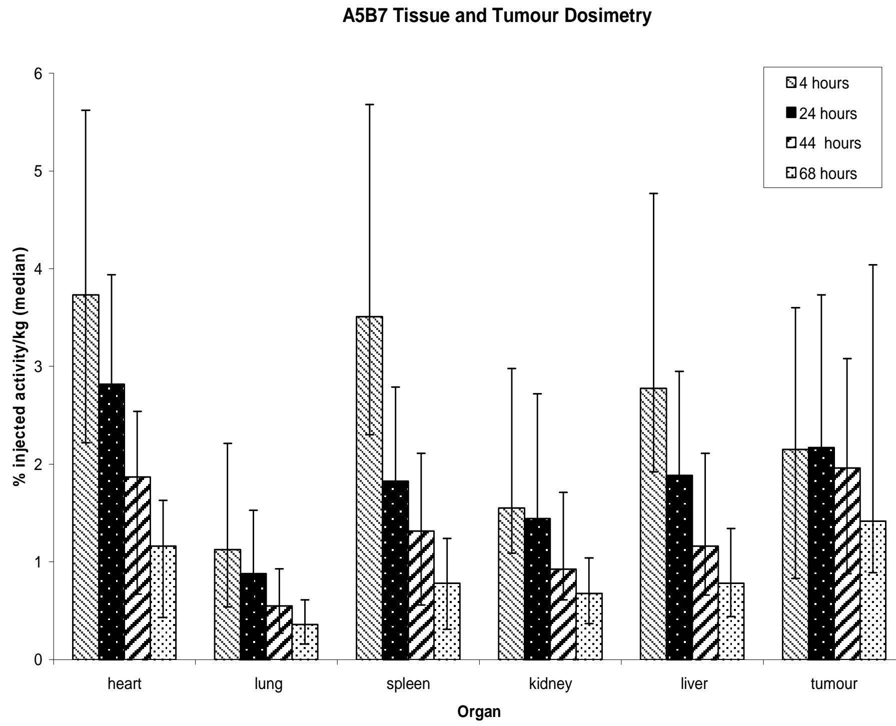


Figure 3.2 Median absorbed radiation doses to normal organs

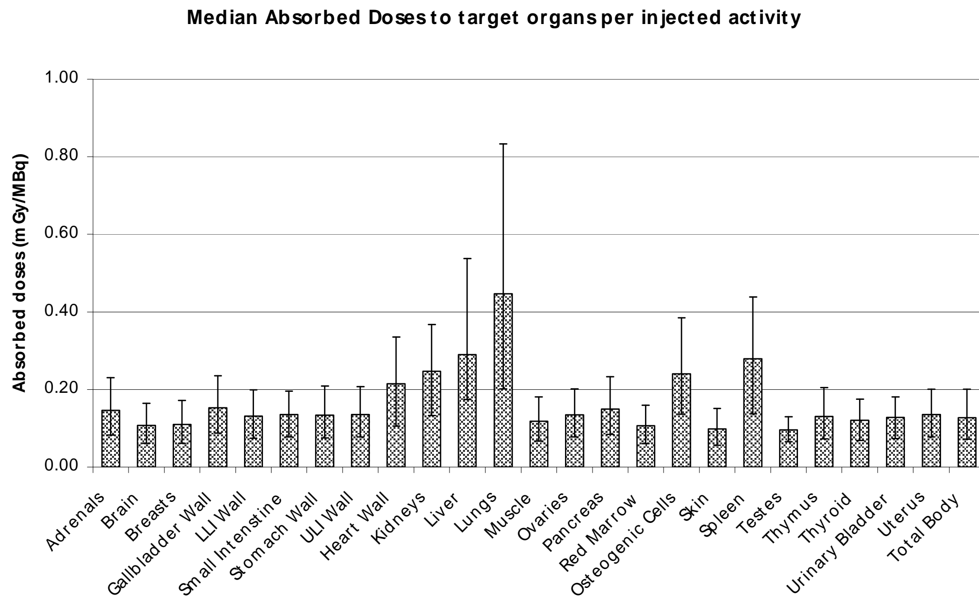
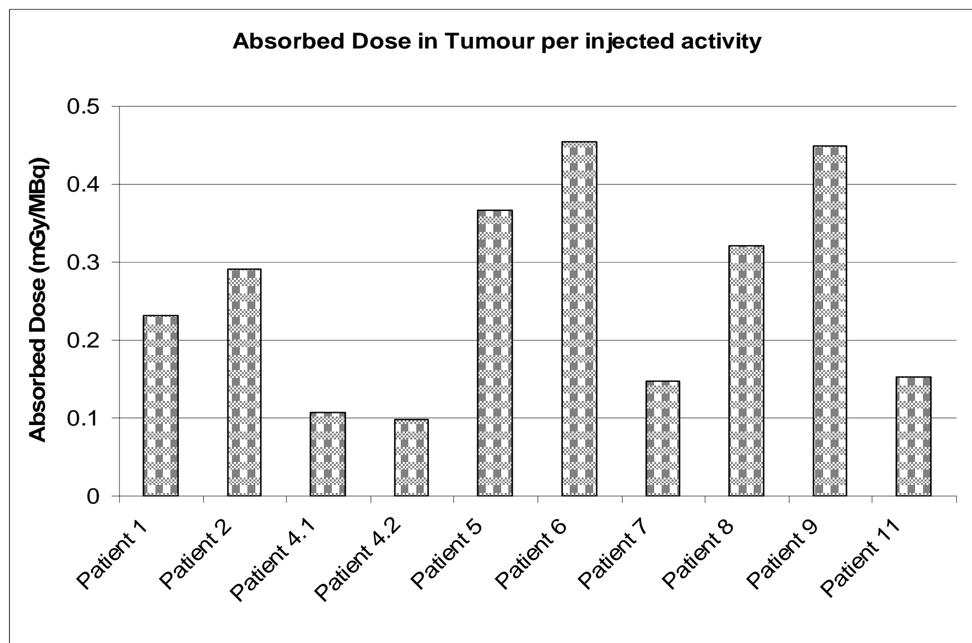


Figure 3.3 Median absorbed radiation dose to tumour



3.6.6 Pharmacokinetics

Pharmacokinetic analysis was performed for both CA4P and ^{131}I-A5B7. The CA4P data was obtained after the 1st infusion and included both CA4P and its metabolite CA4. The conversion of CA4P to CA4 occurred rapidly with an initial half-life of < 10 minutes. CA4 itself was then cleared from the plasma with a terminal half-life of 2 hours. Over the relatively narrow dose-range of CA4P in this study the clearance, half-life and volume of distribution were independent of the dose received, whereas peak concentration and the AUC were related to dose. These results are consistent with previous studies (265, 266).

In 9 patients, the blood clearance of ^{131}I-A5B7 could be modelled using a bi-exponential model. Patient 10 withdrew prior to RIT administration, patient 11's data did not fit this model and patient 12 had too few data points. In the 9 evaluable patients an initial rapid clearance was observed with a second slower phase. As the parameters of the model varied widely between patients the time taken to fall to 50% and 90% was calculated; this was a median of 0.8 hours (range 0.3-17.5 hours) and 46 hours (range 2-77 hours) respectively. The clearance is faster than previously reported (94). It is likely the previous mono-exponential model was due to an initial rapid early phase being missed. If the second clearance phases are compared the results are broadly similar; a range of 9.4-28 hours in this study and a mean of 28.6 hours in Lane et al (94).

Table 3.11 Pharmacokinetic data for CA4P at two dose levels											
Patient	**Dose**	**$CA4P_{max}$**	**CA4P $T_{½}$**	**CA4P $T_{½}$**	**CA4P AUC**	**CL**	**Vss**	**$CA4_{max}$**	**CA4 $T_{½}$**	**T_{max}**	**CA4 AUC**
	mg/m^2	μM	h***	h**	$μmol.h.l^{-1}$	$l.h^{-1}$	l	μM	h**	h	$μmol.h.l^{-1}$
2	45	23.8	0.13	0.54	10.1	18.0	3.91	2.13	2.60	0.3	2.51
3	45	13.1	0.06	0.064	4.34	34.3	4.08	2.81	1.60	0.2	1.75
4	45	22.1	0.09	0.30	6.74	36.3	5.48	1.63	4.00	0.2	2.15
5*	45	27.9	0.08	0.28	7.02	30.9	4.95	1.73	2.20	0.2	1.96
6	45	27.8	0.1	0.62	7.88	27.6	5.04	5.41	0.81	0.3	4.04
Mean		**22.9**	**0.09**	**0.36**	**7.22**	**29**	**4.7**	**2.74**	**2.24**	**0.2**	**2.48**
SD		**6.05**	**0.02**	**0.22**	**2.08**	**7.2**	**0.7**	**1.56**	**1.19**	**0.02**	**0.91**
7†*	54	28.9	0.09	0.60	8.91	0.0	0.00	1.39	2.00	0.3	2.04
8*	54	30.6	0.10	0.39	8.89	28.4	5.54	4.11	1.90	0.3	3.10
9	54	31.8	0.07	0.30	7.97	26.6	3.92	2.97	3.20	0.3	4.14
10*	54	35.3	0.06	0.30	8.43	36.2	5.05	4.23	1.60	0.2	2.67
11*	54	28.9	0.09	0.45	8.09	37.1	6.44	4.70	1.60	0.2	4.65
12	54	30.5	0.16	0.67	10.4	25.5	7.34	1.28	1.20	0.2	1.39
Mean		**31.0**	**0.09**	**0.45**	**8.78**	**26**	**4.7**	**3.11**	**1.92**	**0.2**	**3.00**
SD		**2.4**	**0.03**	**0.15**	**0.88**	**13**	**2.6**	**1.49**	**0.69**	**0.07**	**1.24**

† 18 min infusion, * extrapolated back to end of infusion from fit, ** terminal half-life, *** initial half-life, § Data from patient 1 missing due to contamination of samples

AUC; area under the plasma concentration curve, CL; clearance, Vss; steady-state volume of distribution, T_{max}; time to reach C_{max}

Table 3.12 Pharmacokinetic data for ^{131}I-A5B7							
Dose level ^{131}I-labelled ^{131}I-A5B7 (MBq/m^2)	**Patient number**	**A_{0a} (%ID/kg)**	**A_{0b} (%ID/kg)**	**$T_{½a}$ (h)**	**$T_{½b}$ (h)**	**50.0% (h)**	**10.0% (h)**
1800	1	25.3	12.0	0.4	11.5	0.6	19.4
1800	2	11.5	9.2	0.2	21.5	0.5	46.0
1800	3	72.9	21.3	0.9	14.9	1.2	17.5
1800	4	87.3	11.6	0.3	9.4	0.3	2.0
1800	5	6.6	11.6	9.3	26.6	17.5	71.0
1800	6	23.3	14.7	0.4	28.0	0.8	54.5
1600	7	8.6	10.6	10.0	23.0	15.0	59.0
1600	8	38.5	4.6	0.4	22.3	0.5	3.0
1600	9	4.3	12.2	1.3	26.8	15.0	77.0
	Median					**0.8**	**46**

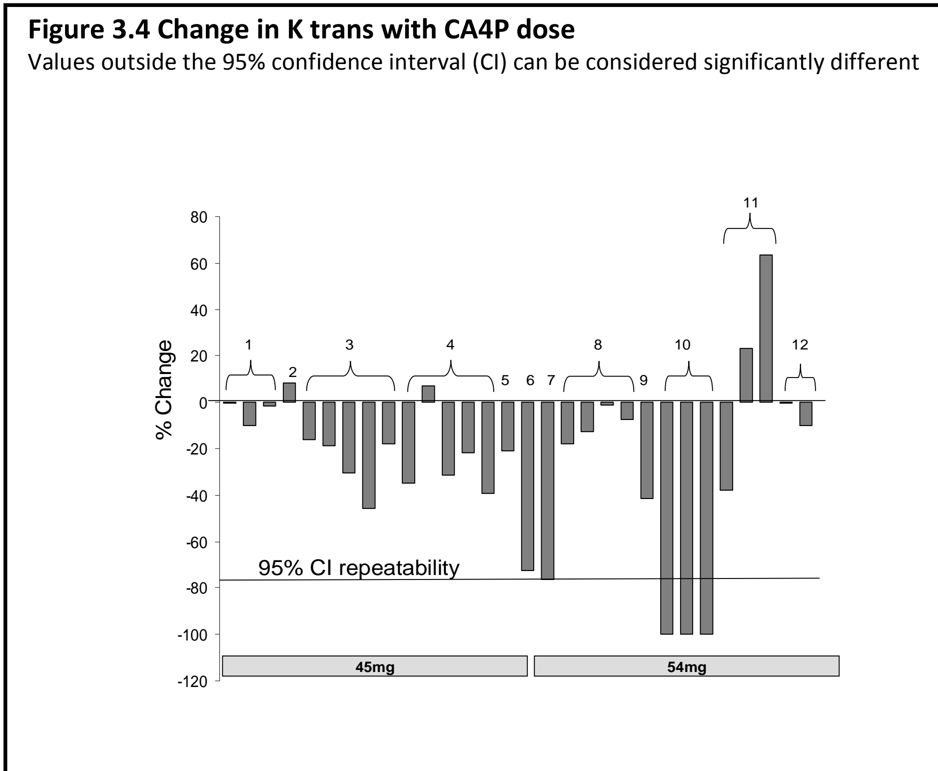
A_{0a} amplitude of the 1st component of the bi-exponential clearance model, A_{0b} amplitude of the 2nd component of the bi-exponential clearance model, $T_{½a}$ Half life of the 1st component of the bi-exponential clearance model, $T_{½b}$ Half life of the 2nd component of the bi-exponential clearance model, %ID/kg; Percentage of injected dose per kilogram, 50% and 10% time taken for the modelled activity to fall to 50 or 10% of it's initial value.

3.6.7 Dynamic contrast enhanced MRI

Patients had two MRI's scans prior to study entry to assess intra-patient variability and a further MRI 4 hours post CA4P to study vascular change. In all, 30 lesions were analyzed. By visual examination 9 lesions had a reduction in central enhancement at 4 hours. By objective measurement 3 patients (patients 6, 7 and 10) had a significant or borderline reduction in both $IAUGC_{60}$ and K^{trans}.

Of the individual lesions assessed 3 of 30 had a significant or borderline reduction in $IAUGC_{60}$ and 5 of 29 had reductions in K^{trans} (see Figure 3.4).

Figure 3.4 Change in K trans with CA4P dose
Values outside the 95% confidence interval (CI) can be considered significantly different



The differences can be explained by tumour mobility and non-uniform reductions in central enhancement being easier to assess visually than objectively. The degree of responsiveness to CA4P was highly variable but less so if lesions in a single patient were considered. As the dose was increased to $54mg/m^2$ a trend was demonstrated towards a greater reduction in tumour blood flow.

3.6.8 Immunogenicity

Nine patients developed HAMA after the first treatment with ^{131}I-A5B7. The one re-treated patient (patient 4) became HAMA positive afterwards. Of the 2

remaining patients: patient 12 did not have a post-treatment measure and patient 10 did not receive any ^{131}I-A5B7.

3.7 Conclusions and Discussion

The A5B7/CA4P study explored the combination of RIT with a VDA and has subsequently been published (276). The combination was considered rational due to the differing methods and sites of action within a tumour, as RIT targets the tumour rim and CA4P the poorly perfused centre (124). It was supported by the success of this combination in eradicating colon carcinoma xenografts in an animal model (123). The scheduling of CA4P 48 hours post ^{131}I-A5B7 was designed to increase the RIC retention in tumour and was based on previous work, which demonstrated trapping up to 96 hours post (123).

Although the toxicity profiles were thought to differ and the selected starting doses were below the MTD's from the single agent studies (265-267), dose-limiting myelosuppression occurred in the first cohort. This myelosuppression recurred despite a subsequent dose reduction to 1600 MBq/m^2, considerably lower than the single-agent MTD of 2400 MBq/m^2 (94). This excess toxicity cannot be explained by an increased red marrow absorbed dose as doses were similar between the two studies at 0.028 cGy/MBq in Lane et al (94) compared to 0.01cGy/MBq in our study. In addition no difference was seen in the pharmacokinetics between the two studies to explain such increased toxicity.

An alternative explanation would be that CA4P has an additional cytotoxic effect similar to other microtubule-binding agents (277) that combined with radiation exposure led to a synergistic toxicity to bone marrow. However the single agent studies of CA4P have not demonstrated myelosuppression even at the highest doses of 114 mg/m^2. Two studies combining chemotherapy (paclitaxel and carboplatin) with CA4P did not show an increased risk of myelosuppression

(278, 279). One study did show an increased risk, when the chemotherapeutic (here carboplatin) was administered 1 hour prior to low-dose CA4P (36mg/m^2) (280). This increased toxicity was attributed to a temporary reduction in renal blood flow secondary to CA4P, resulting in an impaired carboplatin clearance.

Alternatively it could represent the more heavily pre-treated patient group now than in the earlier phase I study by Lane et al (94). When that study was completed fewer chemotherapeutics were available as standard therapy with most patients having none or 5-FU (5-Fluorouracil) alone. In our study 9 of 12 patients had 2 or more lines of chemotherapy with a subsequent loss of bone marrow tolerance.

The sample size in this study was too small to assess for efficacy. It was disappointing that no patients had objective responses as seen in the original study by Lane et al (94) of single-agent RIT. This lack of response may be a true indicator of lack of efficacy or due to the more heavily pre-treated patient group or simply reflect the cohort size. Of note the percentage injected activity/kg in tumour in our study was lower than in the single agent study (94) with values of 2.15% at 4 hours versus 4.4% at 4.25 hours in Lane et al (94).

One patient did achieve a minor response with a corresponding decrease of CA19-9 from 10,507 IU/ml to 2,033 IU/ml. This patient had both the highest absorbed tumour radiation dose and a significant decrease in IAUGC post CA4P, being the only patient to have a significant biological response to both agents. The majority of patients failed to demonstrate adequate vascular shut down on DCE-MRI, possibly due to the relatively low dose of CA4P administered. Patient 10, who did achieve the highest K^{trans} recorded, unfortunately did not proceed to the RIC due to severe tumour pain secondary to CA4P.

Scheduling is vital when combining RIT with other agents to minimise toxicity and ensure maximum efficacy. The CA4P was given at 48 hours post RIT to ensure trapping of the RIC in tumour as anticipated by previous animal work (123). CA4P administration prior to RIT is thought to prevent the RIC accessing tumour reducing any likely benefit as occurred when cisplatin was given post-CA4P (281).

In our study CA4P was administered 1-2 weeks prior to ^{131}I-A5B7 to assess pharmacokinetics. As blood flow had previously been shown to normalise 24 hours post-CA4P (262) it is unlikely this administration could impede tumour uptake of the RIC. In support of this no relationship existed between the extent of vascular shut down on DCE-MRI and subsequent tumour uptake determined by dosimetry. The patients with the greatest disruption of blood flow on DCE-MRI did not have a reduced tumour absorbed dose after ^{131}I-A5B7.

Our study demonstrated ^{131}I-A5B7 did specifically target tumours. Tumour uptake was relatively low at 4 hours compared to normal organs but higher at 68 hours, a pattern consistent with the slow uptake of a whole antibody. Tumour localisation was lower than in the single agent ^{131}I-A5B7 study (94). This may be due to loss of CEA antigen expression in this more heavily pre-treated group of patients. Uptake to normal organs, excepting lung, remained low (< 0.3 mGy/MBq). The apparent high absorbed dose to lung may be due to difficulties in attenuation correction in the thorax, a heterogeneous uptake due to the inclusion of blood vessels or the proximity to the heart.

Tumour absorbed dose was likely to be under-estimated in our calculations. For target organs the dose from neighbouring organs is included but this was not possible for tumours where only the self-dose was calculated. Co-registration of SPECT with CT, to allow accurate delineation of size, shape and relative position of the organs and tumour will be important in future. In addition tumour uptake

is known to be very heterogeneous whereas estimated dosimetry calculations assume uniformity. That assumption means dosages to the well-perfused viable area of tumour may be under-estimated. Even with these caveats the tumour absorbed dose in 4 patients was higher than in all normal organs excepting lung.

In conclusion, the combination of RIT and a VDA was found to be unexpectedly toxic and no objective responses were seen. In one patient (patient 6) who experienced both a reduction of blood flow on DCE-MRI secondary to CA4P and a higher than average tumour absorbed dose on dosimetry there was evidence of efficacy with a fall in tumour markers and a minor radiological response.

Improvements in the delivery of RIT and the potency of the VDA may enhance efficacy. Possible improvements include the use of humanized antibodies to allow repeat dosing, antibody fragments such as minibodies, diabodies or scFv's (45) and more potent VDAs such as DMXAA (269). In addition RIT may be directed against better targets such as the more accessible tumour vasculature (282).

Chapter Four: Biomarkers for Vascular Disruptive Agents

In the Introduction I discussed the need for an adequate biomarker to assess the degree of vascular shut down induced by the Vascular Disruptive Agents. A biomarker would ensure that the optimal biological dose is used to generate maximal vessel shut-down and would enhance the integration of the two therapeutic modalities more successfully. I have discussed imaging methods of assessing vascular shutdown, particularly DCE-MRI and PET-CT and considered their advantages and disadvantages. Finally I considered using serum angiogenic cytokines. These have the advantages of being simple to obtain allowing serial measurements and having quantitative validated methods of analysis that should be possible in standard laboratories.

In this study I decided to investigate four angiogenic cytokines, VEGF (Vascular Endothelial Growth Factor), Angiopoietin 1 and Angiopoietin 2, Erythropoietin and basic Fibroblast Growth Factor (bFGF or FGF2). In this chapter I shall initially summarise the physiological role of cytokines in normal angiogenesis before considering each cytokine individually and their role in HCC and post-TAE since this is the model I intended to use. I shall then present the results of assessing angiogenic cytokines in the context of HCC.

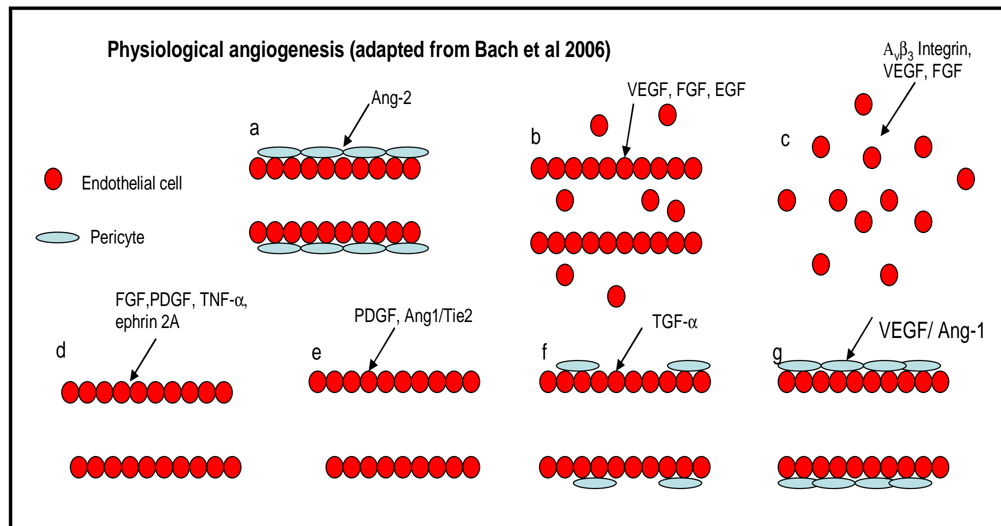
4.1 The role of angiogenic cytokines in angiogenesis

4.1.1 Physiological

Physiological angiogenesis is a tightly regulated process that allows vascular remodelling to occur under the influence of a variety of cytokines. It can either be sprouting or non-sprouting (283). Non-sprouting angiogenesis occurs by vessel splitting via trans-capillary pillars. Sprouting is shown in Figure 4.1. After

pericyte removal secondary to angiopoietin-2 (Ang-2), vessels become leaky with extravasation of proteases. The basement membrane and interstitial matrix dissolves leaving the endothelial cells exposed to cytokines such as VEGF. Endothelial cell proliferation occurs and migration towards angiogenic stimuli with lumen formation. The pericytes are then replaced under the influence of Angiopoietin-1 (Ang-1) to stabilise the new vessel.

Figure 4.1 Physiological Angiogenesis



a) Ang-2 binding to Tie2 on endothelial cells (EC) with subsequent pericyte and smooth muscle removal
b) EC proliferation occurs secondary to cytokines such as VEGF
c) Cell migration occurs secondary to cytokines such as FGF
d) Tube formation
e) Mesenchymal cells are attracted to the ablumenal side of the vessel
f) Cells are induced to differentiate into pericytes
g) Vessel stabilisation. VEGF and Ang-1 are survival factors for EC's

4.1.2 Pathological angiogenesis

Angiogenesis is vital for tumours to develop beyond 1-2mm^3 and to allow invasion and metastasis (284). Pro-angiogenic cytokines are either secreted by the tumour or secondary to tumour hypoxia or change in pH. For example the presence of Angiopoietin-2 in the absence of VEGF leads to tumour vessel regression. Subsequent tumour necrosis and hypoxia generates VEGF secondary to HIF-1α and the combination with Angiopoietin-2 causes rapid angiogenesis.

Tumours may also co-opt existing vasculature or use vascular mimicry where tumour cells act like endothelial cells (285). Endothelial progenitor cells may be recruited from the bone marrow and localise to tumour vessels. The result of this chaotic angiogenesis is a more disorganised vasculature with areas of inadequate blood supply (286).

4.1.2.1 The angiogenic switch hypothesis

This hypothesis considers that the absolute level of any one cytokine is less important than their relationship to other cytokines. When pro-angiogenic cytokines exceed anti-angiogenic ones, there is a ‘switch’ in the system and angiogenesis is favoured. This switch is thought to be essential to the malignant process (287).

4.2 Vascular endothelial growth factor (VEGF)

The first angiogenic cytokine to be discovered was VEGF. The protein was identified in 1983 and initially called ‘vascular permeability factor (VPF)’ due to its role in increasing vessel leakage (288). It was cloned independently by two separate groups (289, 290) and renamed VEGF.

4.2.1 Structure

The VEGF family consists of 6 secreted glycoproteins named VEGF A to E and placental growth factor (PIGF) 1&2 (284). Different isoforms are produced by alternative splicing. Shorter isoforms tend to be secreted whereas the longer are sequestered in the extra-cellular matrix, from where they can be activated by proteases. The commonest isoform is $VEGF_{165}$ which exists in both a soluble and extracellular matrix bound form (287).

VEGF acts via 3 tyrosine kinase receptors and neuropilin-1 and 2. VEGFR-1 (Flt-1) and VEGFR2 (KDR or Flk-1) share 44% homology and are predominantly involved in angiogenesis (287). They are located on endothelial cells and various haematopoietic cell lineages. VEGFR-3 (Flt-4) is predominantly involved in lymphangiogenesis (287).

4.2.2 Function

VEGF is a pivotal protein in angiogenesis and vasculogenesis. Its inactivation in mice leads to early embryonic lethality (291). Post-utero VEGF-A appears necessary for wound healing, ovulation, menstruation, pregnancy and regulation of blood pressure (287). VEGF-B is thought to be redundant; its loss causes no defects in embryogenesis. VEGF C&D are necessary for lymphangiogenesis whilst VEGF-E is exclusively a viral protein (287).

VEGF release is regulated by various genes (e.g. Ras), transcription factors (e.g. HIF) growth factors and cytokines (e.g. IGF-1). Activation of VEGF triggers endothelial cells to proliferate, survive, migrate and differentiate, vascular permeability is enhanced and protein extravasation promoted from blood vessels (284) as well as mobilisation of endothelial progenitor cells from bone marrow.

4.2.3 Tumour expression of VEGF

VEGF is over-expressed in many tumours. Expression is greatest next to areas of necrosis (292), in malignant cell cytoplasm and on associated endothelial cells. High expression correlates with a poorer prognosis and the presence of late stage disease in many cancers including lung, breast, colorectal, gastric, pancreatic, HCC (Hepatocellular cancer), prostate and ovarian cancer (284). In some cancers it is an independent prognostic factor for survival (293) whilst no relationship was found in others (292).

4.2.4 Secreted VEGF

VEGF is found in higher concentrations in the serum or plasma of cancer patients than in healthy individuals (294). It is thought to be tumour-derived as levels fall post surgical resection (294). The level of secreted VEGF have been found to correlate with a poorer prognosis and an advanced stage of disease (284, 295) in breast, small cell lung (where it was an independent prognostic factor), non-small cell lung, colorectal, gastric, HCC, prostate, bladder, renal cell and ovarian cancer (284).

High VEGF levels have predicted a poorer response to chemotherapy (296), radiotherapy and immunotherapy (297). Human circulating VEGF falls on tumour resection, with some evidence of a rise on relapse (284). In gastric cancer VEGF levels could distinguish between a partial response and progressive disease (298).

However other studies have not confirmed that VEGF correlates with prognosis and in many tumour types, including ovarian cancer, the data remain contradictory (299) with little consensus as to the value of VEGF. In HCC a recent meta-analysis of 8 studies found did predict a poorer overall survival and

progression-free survival in patients with an elevated serum VEGF but all but one of those studies was carried out in the Asian population with a differing mix of aetiologies (300). Although VEGF levels are higher in advanced disease in these tumour types (284) it has proved disappointing as a biomarker. Levels of VEGF are variable and often remain low even in advanced disease. When they do rise they often rise relatively late in the disease process, making them unsuitable as a biomarker.

4.2.5 Measuring secreted VEGF – serum or plasma?

Serum VEGF is higher than plasma VEGF as it includes VEGF derived from platelets during coagulation (284). Platelets are thought to scavenge circulating VEGF, thereby restricting it to sites of coagulation (e.g. wounds) and preventing general EPC (Endothelial progenitor cell) recruitment (301). In support of this, platelet VEGF levels are higher in cancer patients (302) and tumour VEGF expression correlates well with the platelet-derived VEGF level (301). George et al used the serum VEGF normalized by the platelet count (303), considering plasma samples potentially more inaccurate as their lower values were closer to the limits of detectability by ELISA.

4.2.6 Angiogenic cytokines in HCC: VEGF

In HCC both tumour and tumour-associated endothelial cells over-express VEGF, the latter also over-express VEGFR-1 (Vascular Endothelial Growth Factor Receptor) and VEGFR-2 (304). Expression is highest at tumour boundaries (305) and correlates with MVD and hypervascularity. In animal models stimulating VEGF led to tumour growth and neo-vascularisation (304). Some human studies correlated VEGF expression with more invasive tumours and a poorer prognosis (145) but other did not. VEGF interacts with other cytokines and Moon et al demonstrated correlation between VEGF protein and Ang-2 mRNA (306).

Serum and plasma VEGF rise in HCC (307). An elevated serum VEGF predicts a poorer outcome post resection (308), TACE (trans-arterial chemo-embolisation) (309) and radio-frequency ablation (RFA) (310). In a meta-analysis, where all but 1 study was in the Asian population, an elevated serum VEGF was associated with a poorer survival (300). Serum VEGF is an independent prognostic factor and associated with other poor prognostic factors such as portal vein thrombosis and advanced tumour stage (307).

4.3 Angiopoietins 1 and 2

Angiopoietins are secreted glycoproteins. Four family members have been identified (Ang1-4) of which Ang-1 and Ang-2 are the best characterized (311) sharing 60% homology. They both interact with the Tie-2 (*t*yrosine kinase with *i*mmunoglobulin and *e*pidermal growth factor homology domains-2) receptor as competitive inhibitors binding on the same site with similar affinity (311). Ang-1 is an agonist whereas Ang-2 is the antagonist so Tie-2 activation depends on the relative balance of Ang-1 and Ang-2.

The Tie-2 receptor is almost exclusively expressed on endothelial cells and haematopoietic cells. As tumour cells express Ang-1 and Ang-2 an autocrine / paracrine feedback loop may exist (312).

4.3.1 Angiopoietin-1 (Ang-1)

4.3.1.1 Structure of Angiopoietin-1

Alternate gene splicing produces 3 forms of angiopoietin-1 but only one can activate Tie-2. It exists in a coiled-coil structure allowing dimerization and super-clustering into heterogeneous multimers. At least 4 subunits are required to

activate Tie-2 (313) but larger clusters are less effective. The coiled-coil structure can bind and inactivate other angiopoietins.

Angiopoietin-1 is widely expressed in many cell types and tissues including peri-endothelial cells (smooth muscle and pericytes), fibroblasts, megakaryocytes and platelets (311). It is constitutively expressed in healthy individuals (314).

4.3.1.2 Function of angiopoietin-1

Ang-1 has a central role in promoting blood vessel development, remodelling and maturation, acting in a paracrine manner (315). It enhances endothelial cell survival, quiescence and maturation, encourages recruitment of peri-endothelial cells (pericytes and smooth muscle cells) and localization of cell adhesion molecules leading to vessel stability (316, 317). It reduces vessel permeability and is anti-inflammatory. In cell line work Ang-1 promotes endothelial cell migration, tube formation and cell survival but not proliferation (318).

Knock-out of Ang-1 in mice leads to early embryonic death. Vasculature is abnormal, lacking complexity and with poor association between endothelial and support cells (319). Post embryogenesis Ang-1 is thought to maintain the mature quiescent vasculature. The relationship with Ang-2 appears context dependent and in the brain Ang-1 appears to increase vascularisation in synergy with VEGF (320).

4.3.2 Angiopoietin-2 (Ang-2)

4.3.2.1 Structure of Angiopoietin-2

Angiopoietin-2 also has a coiled-coil structure and a fibrogen like domain found in other proteins such as fibrinogen and tenascin (311). It interacts with the Tie-2 receptor in an autocrine manner (314) and its expression is tightly regulated. Ang-2 is secreted by endothelial cells at sites of vascular remodelling with secretion induced by shear stress, hypoxia and VEGF release. Ang-2 may be stored in specialized endothelial-cell granules, the Weibel-Palade bodies and secreted as required (162).

4.3.2.2 Function of Angiopoietin-2

Ang-2 binds to Tie-2 and prevents Ang-1 from activating it (311). Ang-2 is vital for vascular remodeling. In the presence of VEGF vascular sprouting and angiogenesis occur, but in its absence there is vascular regression such as in atretic follicles (319). Ang-2 generates a destabilized vasculature with dissociated support cells and greater access for cytokines such as VEGF.

Mice deficient in Ang-2 appear to develop normally in-utero (except those with C129 genetic backgrounds) but die within 2 weeks of birth (321). Abnormalities are seen in the neonatal eye and in post-natal lymphatics. Mice with over-expressed Ang-2 have similar defects (and embryonic lethality) to those with knocked out Ang-1 or Tie-2 (322). The normal expression of Ang-2 is restricted post-natally to sites of vascular remodelling, the ovaries, uterus and placenta (323). Ang-2 levels increase up to 15-fold with hypoxia (324).

4.3.3 Summary of angiogenic cytokines in various vessel states

Bach et al considered how the effects of a single cytokine depend on the values of other angiogenic cytokines (325)(see Table 4.1).

Table 4.1 Summary of angiogenic cytokines in various vessel states

	Maturation	Regression	Proliferation
Ang-1	High	Low	Low
Ang-2	Low	High	High
VEGF	High	Low	High

4.3.4 Angiopoietins and Cancer

4.3.4.1 Angiopoietin-2

Colorectal and gastric cancer cells transfected to over-express Ang-2 and grown in nude mice generate larger, more vascular and more invasive tumours that are more likely to metastasize (325, 326) than controls. Blocking Ang-2 led to tumour stasis or response (325). Combining Ang-2 with VEGF in a murine model of HCC decreased apoptosis and increased MMP (matrix metallopeptidase)-2 and 9 rather than enhancing tumour cell proliferation (327). The pro-angiogenic effect of Ang-2 on tumours appeared more important in early development than their later stages (328).

Ang-2 expression is elevated in many human cancers (see Table 4.2) including renal, gastric, breast, angiosarcoma, colon, pancreatic (neuroendocrine and adenocarcinoma), HCC and NSCLC (Non-small cell lung cancer) (325). Expression appears greatest in the more vascular tumours and adjacent to areas of hypoxia and necrosis. The Ang-2 source may be the tumour-associated endothelial cells rather than tumour cells (314). Its over-expression is often associated with a more advanced stage and a poorer prognosis.

Table 4.2 Tumour over-expression of Angiopoietin-2		
Tumour type	Association Ang-2 over-expression	Reference
Gastric	Increased stage and vascular involvement. Poorer survival	Etoh et al (246)
Breast	Lymph node spread, shorter PFS and poorer survival. Correlated with Her-2 over-expression	Sfiligoi et al (249)
HCC	Poorer survival, increased MVD and tumour size.	Mitsuhashi et al (250)
NSCLC	Increased stage. Poorer survival (especially if high VEGF). Independent prognostic factor	Takanami et al (251) Tanaka et al (252)
Bladder	Poorer survival and worse grade and stage	Oka et al (253)
Prostate	Higher Gleason score, increased risk of metastatic disease, poorer survival	Lind et al (254)
Colorectal	Ang-2 expression higher in metastatic disease.	Tait et al (244)

† PFS: Progression Free Survival

4.3.4.2 Angiopoietin-1

Ang-1 over-expression in tumour-bearing mice leads to smaller tumours, fewer and more mature tumour-associated blood vessels with a greater coverage by peri-endothelial cells and a reduced permeability (325).

Ang-1 expression is highly variable between tumour types and its role in tumour angiogenesis somewhat controversial (316). Many tumour types express high levels of Ang-1, which theoretically should reduce angiogenesis and tumour growth (314, 329, 330). High Ang-1 expression in renal cancer was associated

with low-grade tumours and a better prognosis. Other studies have found no differences in Ang-1 expression between tumour and normal tissue (324, 331). A reduced Ang-1 (and normal Ang-2) expression has been found in ovarian (332) and colorectal cancer (333).

4.3.4.3 Ratio of Angiopoietin-2 / Angiopoietin-1

As the angiopoietins are competitive inhibitors their ratio might be more accurate than their individual levels. In HCC a high Ang-2/Ang-1 ratio was associated with a larger tumour size, portal vein thrombosis and a higher MVD (334). An increased ratio is seen in NSCLC (335), prostate cancer, oral squamous cell cancer and ovarian cancer (325) amongst others (see Table 4.3).

Table 4.3 Ratio of angiopoietin expression in tumour tissue Adapted from Tait et al (244)				
Tumour	**Ang-1**	**Ang-2**	**Ratio Ang2:Ang1**	**Reference**
Breast	Up	Up	Up	Sfiligoi et al (249)
	Down	Down	Up	Currie et al (261)
	Up	Up	Not clear	Canine et al (262)
HCC	No change	Up	Up	Tanaka et al (257)
	No change	Up	Up	Mitsuhashi et al (250)
	Up	Up	Up	Moon et al (263)
	Up	Up	Up	Sugimachi et al (264)
Colon	No change	Up	Up	Yoshida et al (265)
	No change	Up	Up	Ahmad et al (259)
	No change	Up	Up	Ogawa et al (266)
Lung	Down	Up	Up	Wong et al (260)
	Up	Up	Not clear	Tanaka et al (252)
	Up	Up	Not clear	Hatanaka et al (267)
Gastric	No change	Up	Up	Etoh et al (246)
Ovarian	No change	Up	Up	Zhang et al (268)
	Down	No change	Up	Hata et al (258)
Melanoma	No change	Up	Up	Pomyje et al (269)
Renal	No change	Up	Up	Currie et al (270)

4.3.4.4 Secreted angiopoietins in cancer

Serum angiopoietin-2 levels are higher in many cancer patients than in normal healthy controls. An elevated angiopoietin-2 is present in ovarian cancer (336), colorectal cancer (337), gastric cancer (338), cervical cancer (339), melanoma (340), neuroendocrine tumours (341) and lung cancer (342). It is often associated with poor prognostic clinico-pathological features and may itself be

an independent prognostic factor (337). In colorectal cancer patients given bevacizumab, a low serum Ang-2 was associated with better response rates, longer progression-free survivals and a reduction of 91% in the hazard of death (343).

Secreted angiopoietin-1 has been less extensively studied. In many tumour types it is not significantly different to healthy controls (341, 344, 345). Park et al found a reduced serum Ang-1 level in lung cancer and a negative correlation with MVD. An elevated Ang-1 was associated with an improved disease specific and relapse free survival (342).

4.3.4.5 Angiogenic cytokines in HCC: Angiopoietins

Ang-1 and 2 are expressed in HCC tumour cells with Ang-2 present in the most poorly differentiated tumours and adjacent to areas of cirrhosis (305, 306, 331, 346). In some studies Ang-2 is higher in HCC cells, in others in the adjacent cirrhosis (334). Zeng et al reported Ang-2 expression as similar between normal liver, cirrhotic liver and HCC on a background of cirrhosis (347). The reason for these discrepant findings remains unclear although it may relate to tumour heterogeneity with expression profiles of the tumour rim differing from the hypoxic centre.

Ang-2 expression in HCC correlates with a more aggressive tumour grade, poorer prognosis (305), an increased vascularity and an earlier recurrence (346). Ang-1 expression had no impact on prognosis or survival (346). Wada et al found both Ang-1 and Ang-2 expression were elevated in HCC. Patients with a high Ang-2: Ang-1 ratio had a worse prognosis and a higher MVD (334).

Scholz et al (348) found serum Ang-2 was elevated in cirrhosis and further elevated in HCC. The addition of Ang-2 to AFP (alpha fetoprotein) improved the

diagnosis of HCC in patients with cirrhosis. Serum Ang-2 did not correlate with clinico-pathological characteristics in this retrospective study, unlike in the tissue expression data.

4.4 Basic fibroblast growth factor (bFGF)

FGF-2 (basic FGF or heparin binding growth factor-2) belongs to a large family of 18 fibroblast growth factors that are grouped into 6 subfamilies with sequence homology of 30-50% (349).

4.4.1 Structure of b-FGF

Basic FGF has a core region of 120-130 amino acids in 12 anti-parallel β–strands. It contains the heparan sulphate glycosaminoglycan binding site, heparan sulphate is necessary for successful signaling (350) via trans-membrane tyrosine kinase receptors (349).

4.4.2 Function of b-FGF

The function of bFGF remains controversial. If the bFGF gene is knocked out in mice, vascular tone is reduced and there is a weaker response to vasoconstrictors, with some reduction in cortical neurones (351) but the mice are viable. bFGF may have a role in inflammation leading to smooth muscle hyperplasia (352). It causes fibroblast proliferation and can stimulate angiogenesis by encouraging endothelial cell migration and proliferation, tube formation and disruption of the basement membrane (353). Basic FGF appears to act synergistically with VEGF (354).

4.4.3 B-FGF in cancer

Basic FGF is over-expressed in many cancers (355), including lung, colon, gastric, pancreatic, HCC, bladder and head and neck (284). In some studies bFGF was associated with more advanced disease and a poorer prognosis but others showed no difference (284, 356). An elevated b-FGF may be associated with an improved prognosis as more aggressive tumours may lose b-FGF-expressing myoepithelial cells. In ovarian cancer B-FGF appears to promote fibroblast production and more indolent tumours (357).

Serum bFGF is elevated in some cancer patients and is thought to be tumour derived (284). Its significance is unclear. In breast cancer no correlation could be determined between elevated serum bFGF and prognosis or tumour stage (358). In NSCLC patients on radiotherapy an elevated bFGF was associated with a better outcome, attributed to more fibrosis (359). Results in colorectal and prostate cancer were inconclusive (284). Studies in HCC and renal cancer had a positive correlation between high serum bFGF and advanced stage (284, 360). In head and neck cancer serum bFGF predicted survival post chemo-radiotherapy and was an independent prognostic marker (361). In cervical cancer bFGF rose prior to disease relapse and is being investigated as a biomarker (362).

4.4.3.1 Angiogenic cytokines in HCC: bFGF

bFGF expression is elevated in HCC compared to normal or surrounding liver (350) and correlates with MVD but not hypervascularity as determined by angiography (363). Poon et al found a correlation between serum bFGF and risk of recurrence post resection (364) but this has not been confirmed (365). Serum bFGF could be correlated with advancing stage in HCC but values were not different from patients with Cirrhosis alone (360).

4.5 Erythropoietin

Erythropoietin (Epo) is a glycoprotein hormone (366) produced mainly in the foetal liver and adult kidney. It acts to regulate the production of red blood cells by the bone marrow, acting via its specific receptor EpoR (Erythropoietin receptor).

4.5.1 Structure of Erythropoietin

Erythropoietin is comprised of 165 amino acids. Its receptor is a member of the type I cytokine receptor super-family that includes receptors for many interleukins and haematopoietic growth factors (367). EpoR expression is tightly regulated with its mRNA (messenger ribonucleic acid) having a short half-life of 90 minutes (368).

4.5.2 Erythropoietin Function

Erythropoietin secretion is stimulated by hypoxia (HIF-1 (368)), hypoglycaemia, intracellular calcium, insulin, oestrogen, androgenic steroids and other cytokines (369). Erythropoietin stimulates erythroid cells to proliferate and differentiate into mature cells and reduces apoptosis thereby increasing blood haemoglobin (370). Mice with the erythropoietin gene knocked out die in-utero with abnormal liver erythropoiesis, angiogenesis defects and increased apoptosis of endothelial and myocardial cells (371). Activating mutations cause a primary erythrocyotosis.

Erythropoietin is expressed on erythrocytes but also stem cells in the embryonic nervous system, uterus and ovary (372). It may protect cells from ischaemia and toxic stress (368) via an anti-apoptotic mechanism (373). EpoR is found on endothelial cells with activation causing endothelial cell proliferation and

migration in vitro (374). Exogenous Epo can lead to in vivo neo-vascularisation in chick embryos (375).

4.5.3 Erythropoietin in Cancer

The exogenous administration of erythropoietin in cancer patients increases haemoglobin levels and improves quality of life (376) but two randomized studies found patients on Epo had a poorer outcome (377, 378) possibly due to stimulation of cancer growth. A third randomised study found any stimulatory effect was only present in tumours that expressed EpoR (378).

An elevated EpoR expression is found in many tumour types as shown in Table 4.4 but expression may not indicate receptor activity. Activity can be demonstrated by downstream signalling and proliferation secondary to exogenous Epo. Downstream signalling has been identified in cell lines (379) although with low levels of proliferation. Blocking signalling in xenografts reduced tumour vascularity (368), tumour cell survival and angiogenesis but had no effect on red blood cell formation. EpoR activation has been detected in melanoma (380), uterine and cervical carcinoma (381). In contrast, four studies of exogenous Epo in cell lines expressing EpoR found no increase in tumour cell proliferation (382-385) and no signal transduction.

In patients exogenous Epo improved tumour oxygenation independently of haemoglobin (386), which may reduce the hypoxic stimulus for cytokine release. Sensitivity to chemotherapy and radiotherapy increased with no increase in MVD or tumour growth in animal models (387, 388). Therefore the poorer results in the randomized studies of exogenous Epo remain controversial.

Table 4.4 Erythropoietin and Erythropoietin Receptor Tumour Expression
Table adapted from Hardee et al (316). It details whether either Erythropoietin (Epo) or the Erythropoietin Receptor (EpoR) were found expressed on tumour cells. The methods used are also identified in column 2. IHC was expressed semi-quantitatively in these studies.

Primary tumour	**Method used**	**Expression of:**	**Reference**
Breast	IHC	Epo^+ and $EpoR^+$	Acs et al (309)
Melanoma	IHC	Epo^+ and $EpoR^+$	Kumar et al (310)
Cervical SCC Cervical ADC	RT-PCR RT-PCR	Epo^+ and $EpoR^+$ $EpoR^+$	Yasuda et al (317) Yasuda et al (317)
Endometrial carcinoma	IHC	Epo^+ and $EpoR^+$	Acs et al (318)
Ovarian ADC	RT-PCR	Epo^+ and $EpoR^+$	Yasuda et al (317)
Gastric ADC	IHC	$EpoR^+$	Ribatti et al (319)
Colon tumour	RT-PCR, WB	$EpoR^+$	Arcasoy et al (320)
Head and neck	IHC	Epo^+ and $EpoR^+$	Arcasoy et al (321)
Lung ADC and SCC	RT-PCR, IHC	Epo^+ and $EpoR^+$	Dagnon et al (322)
Prostate ADC	IHC	Epo^+ and $EpoR^+$	Acs et al (311)

IHC: Immunohistochemistry; RT-PCR: Reverse transcription polymerase chain reaction; WB: Western blot

4.5.3.1 Angiogenic cytokines in HCC: Erythropoietin

In a murine model of HCC erythropoietin expression was higher in tumour cells than cirrhotic ones and absent in mice with cirrhosis without HCC (372). In human HCC, Epo and EpoR were strongly over-expressed on tumour-associated endothelial cells with weaker expression on sinusoidal cells in adjacent cirrhosis (389). Epo/EpoR expression was found to positively correlate with tumour grade and vascularity.

Erythrocytosis is present in 2-12% of patients with HCC and increases with advancing tumour stage. It is associated with an elevated serum erythropoietin (390, 391).

4.6 Cytokines post TAE

As discussed in the Introduction we decided to use a model to represent vascular shut-down. The model we chose was Trans-arterial embolisation (TAE). TAE has the advantage of a more definitive time of vascular shut-down and there are greater numbers of patients undergoing this procedure compared to the small numbers of patients on a Phase I study of a VDA.

It is known that TAE leads to profound tumour hypoxia which would be anticipated to stimulate pro-angiogenic cytokine release (392). Indeed this may be an important mechanism by which tumour vasculature recovers post TAE (393).

In an animal model of TACE, both VEGF and HIF-1α were observed to rise post-procedure by day 7 (394). This rise in VEGF could be attenuated by blocking HIF-1α transcription. Shim et al found serum VEGF rose at day 1-2 post TACE returning to baseline by day 21 (395) and was an independent prognostic factor in HCC. If VEGF increased by > 50% post TACE there was a higher risk of extra-hepatic disease within 6 months and a poorer prognosis (308). Post TACE the highest VEGF expression is at the boundaries between necrotic and viable tissue. bFGF is not known to rise post TAE and there is no data on the angiopoietins and erythropoietin.

4.7 Methods

In this study we analysed serum concentrations of VEGF, Ang-1 and -2, bFGF and erythropoietin in a cohort of patients with HCC and two control cohorts, one with cirrhosis and the other healthy controls. We performed univariate and multivariate analysis to identify factors predictive of survival and, in a subset, explored changes in serum cytokine levels before and after trans-arterial embolisation.

4.7.1 Sample collection and analysis

Venous blood (6ml) was collected in serum separator tubes, kept at room temperature and allowed to clot for 30 minutes being centrifuged at 2400rpm for 15 minutes. They were aliquoted into 100µl portions and stored at -80˚C until analysis. Freeze-thaw cycles were avoided.

Serum cytokine concentrations were determined by the relevant Quantikine Immunosorbent assay kit according to manufacturer's instructions. The Ang-1, Ang-2 and VEGF assays were all Quantikine®, the Erythropoietin was Quantikine® IVD® and the FGF Basic assay was Quantikine® HS (R&D systems, Minneapolis, MN). These assays have previously been shown to be reproducible. They are quantitative sandwich immunoassays using an immobilized murine monoclonal antibody and a second monoclonal or polyclonal antibody, both to react with the relevant cytokine and having no cross-reactivity to other cytokines. The cytokine concentration was determined by interpolation to a standard curve generated from known standard concentrations of cytokine. Face-masks were worn to prevent contamination of the samples as VEGF, Ang1&2 and bFGF (alkaline phosphatase) are present in saliva. All samples were assayed in duplicate. A typical method is described below.

VEGF

A known VEGF (recombinant $VEGF_{165}$) concentration (2000 pg/mL) was used to create a dilution series. 100 μL of sample was used per well (coated with murine monoclonal anti-VEGF antibody) and 100 μL of assay dilutent (buffered protein base). Incubation was for 2 hours at room temperature after which the plate was aspirated and washed using a multi-channel pipette. A minimum of 6 washes was performed after which the plate was inverted against paper towels to dry. 200 μL of VEGF conjugate was added (polyclonal anti-VEGF antibody conjugated to horseradish peroxidase) and incubated and washed as described above. 200 μL of substrate was added (50:50 mix of hydrogen peroxide and chromogen; mixed within 15 minutes of use and protected from light). The plate was incubated for 25 minutes in the dark before 50 μL of 2N sulfuric acid was added to stop the reaction. The plate was read with a microplate reader at 450nm and wavelength correction at 550nm. Values could then be read from a standard cure generated from the dilution series. Differences in methodology with the other cytokines are detailed below.

Differences with the other cytokines

- Patient serum was diluted (15 fold for Ang-1 and 5-fold for Ang-2). When the concentration was read from the standard curve it was multiplied by the dilution factor
- A horizontal orbital microplate shaker (500 +/- 50rpm) was used for incubation for Ang1 &2
- The antibody conjugate used monoclonal antibodies for Ang1&2 conjugated to horseradish peroxidase, polyclonal for erythropoietin and conjugated to alkaline phosphatase for bFGF.
- The incubation times differed slightly between cytokines
- The substrate for bFGF used lyophilized NADPH (Nicotinamide adenine dinucleotide phosphate) with amplifier.

- The plate for erythropoietin was read at 450nm with correction at 600nm and 490nm / 650nm for bFGF

4.7.2 Statistical analysis

The data was analyzed using GraphPad Prism 5 software (Software, San Diego, CA). Cytokine values were compared between the groups using a Mann-Whitney U test. Data was expressed using median values. The Kaplan-Meier method was used to estimate overall survival curves and survival curves were compared using the log-rank test. For univariate and multivariate analysis all variables are analysed as continuous variables other than sex, ascites, and alcohol and viral aetiology which were categorical.

As a small pilot study formal power calculations were not undertaken prior to collecting samples. This was due to the likely anticipated small sample size. Due to the relative rarity of patients undergoing TAE or TACE for hepatocellular cancer – it was anticipated that collecting data from approximately 50 patients would take 2 years. For a pilot study it would be unfeasible to collect data for longer durations. However as a pilot study it was accepted that it would not be powered to allow definitive comparisons between groups but only allow identification of promising cytokines for future investigation. Assessing how our data fits in with other groups data sets (that are themselves small) will also aid our selection of promising cytokines for future study.

4.8 Results

4.8.1 Patient characteristics

Between October 2006 and June 2008, serum was collected from 46 patients with HCC and 51 patients with cirrhosis attending the oncology and hepatology clinics at the Royal Free Hospital in London. The number of samples collected for this pilot study was determined by the number of patients visiting the oncology HCC clinic of a period of 2 years. As a pilot study to determine the feasibility of the approach no power calculations were done in advance. As such it is not powered to make definitive direct comparisons but instead to consider promising cytokines to investigate further.

In addition samples were taken from 50 healthy controls including staff volunteers. Inclusion criteria for the patients with HCC were age over 18 years and a diagnosis of HCC based on histology or the criteria defined in the American Association for the Study of Liver Diseases (AASLD) guidelines (396). Evidence of cirrhosis was determined either radiologically or histologically. The study was approved by the Local Ethics Committee and written informed consent was obtained for all subjects.

Patient characteristics are shown below in Table 4.5. Most patients with HCC were male, had a background of hepatitis C infection and were in Child-Pugh class A. They had multifocal disease confined to the liver with a maximum tumour dimension of 20-50mm. The cirrhotic control group had a lower proportion of patients with hepatitis C but otherwise there were no notable differences with the two groups.

Table 4.5 Patient Characteristics
* Signs of portal hypertension included splenomegaly, varices and collateral vessel formation and ascites.

	Characteristics	Patients with HCC (n = 46)	Patients with Cirrhosis (n = 51)
Age (years)	Mean (range)	61 (38-79)	57 (21-76)
Sex	Male Female	37 9	34 17
Aetiology (some patients have more than 1 aetiology)	HBV† HCV‡ Alcohol Autoimmune* Haemochromatosis NASH* Idiopathic	5 27 10 2 2 3 5	2 24 13 4 2 3 4
Albumin (g/dL)	Median (range)	38 (24-50)	37 (27-51)
Bilirubin (μmol/L)	Median (range)	18 (6-67)	24 (6-136)
Serum αFP (μg/L)	Median (range)	21 (2-100000)	3.3 (2-55)
Platelets ($x10^9/L$)	Median (range)	125 (43-349)	109 (20-430)
Child-Pugh score	A B C	29 16 1	34 16 1
Maximum diameter (1 patient no scan available	Median(range) mm ≤ 2cm (no patients) 2-5cm > 5cm	34 (10-140) 5 26 14	N/A
Staging	I II IIIa IIIb	4 8 5 2	N/A

	IVa IVb	23 3	
Distribution	Unifocal Multifocal	19 27	N/A
Portal hypertension**	Yes: No:	29 14	N/A
Therapy	Embolisation Chemo-embolisation Other	25 10 11	N/A
Okuda stage (397)	One Two Three	22 15 9	N/A
CLIP score (398) (Cancer of the liver Italian program)	Early Intermediate Advanced	10 31 5	N/A

† Hepatitis B virus; ‡ Hepatitis C virus * Non-alcoholic Steatohepatitis

Thirty three patients had treatment with TAE or TACE and further serum samples were collected at defined time points following treatment to assess changes in serum cytokine levels. In order to reliably control for the angiographic procedure involved in TAE, a proportion of the healthy volunteers were selected from those undergoing routine cardiac angiography provided they had had no recent severe chest pain, recent diagnosed myocardial infarction, elevated cardiac enzymes and were known not to have pulmonary hypertension or chronic inflammatory conditions.

4.8.2 Baseline cytokine values

Baseline cytokine values (median and dot plot) are provided in Figure 4.2. Patients with HCC or cirrhosis have higher serum Ang-2 and erythropoietin levels than healthy controls. Patients with HCC or cirrhosis also had lower serum Ang-1 levels and therefore a higher ratio (Ang-2 / Ang-1) compared to healthy controls. There was no difference between patients with HCC and healthy controls with respect to VEGF and bFGF.

Comparing the HCC and cirrhotic control groups, Ang-2 tended to be higher and Ang-1 lower in HCC patients compared with cirrhotics but the difference was non-significant. Similarly there were no significant differences between VEGF levels and Erythropoietin between the groups. For bFGF, the concentration was lower in HCC than cirrhotics which was also lower than normal healthy controls but statistical significance was not reached.

Figure 4.2 Baseline Cytokine Values

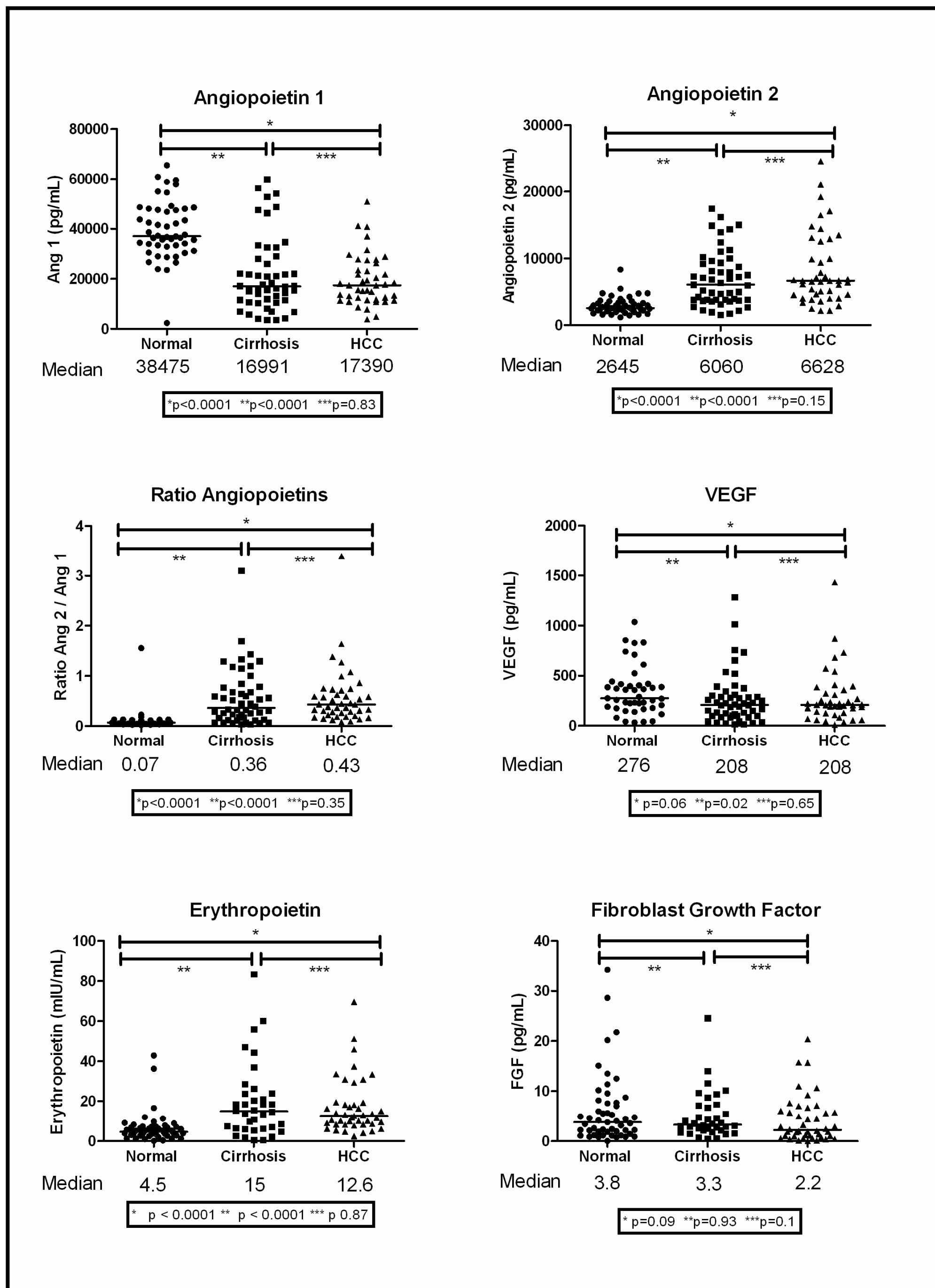
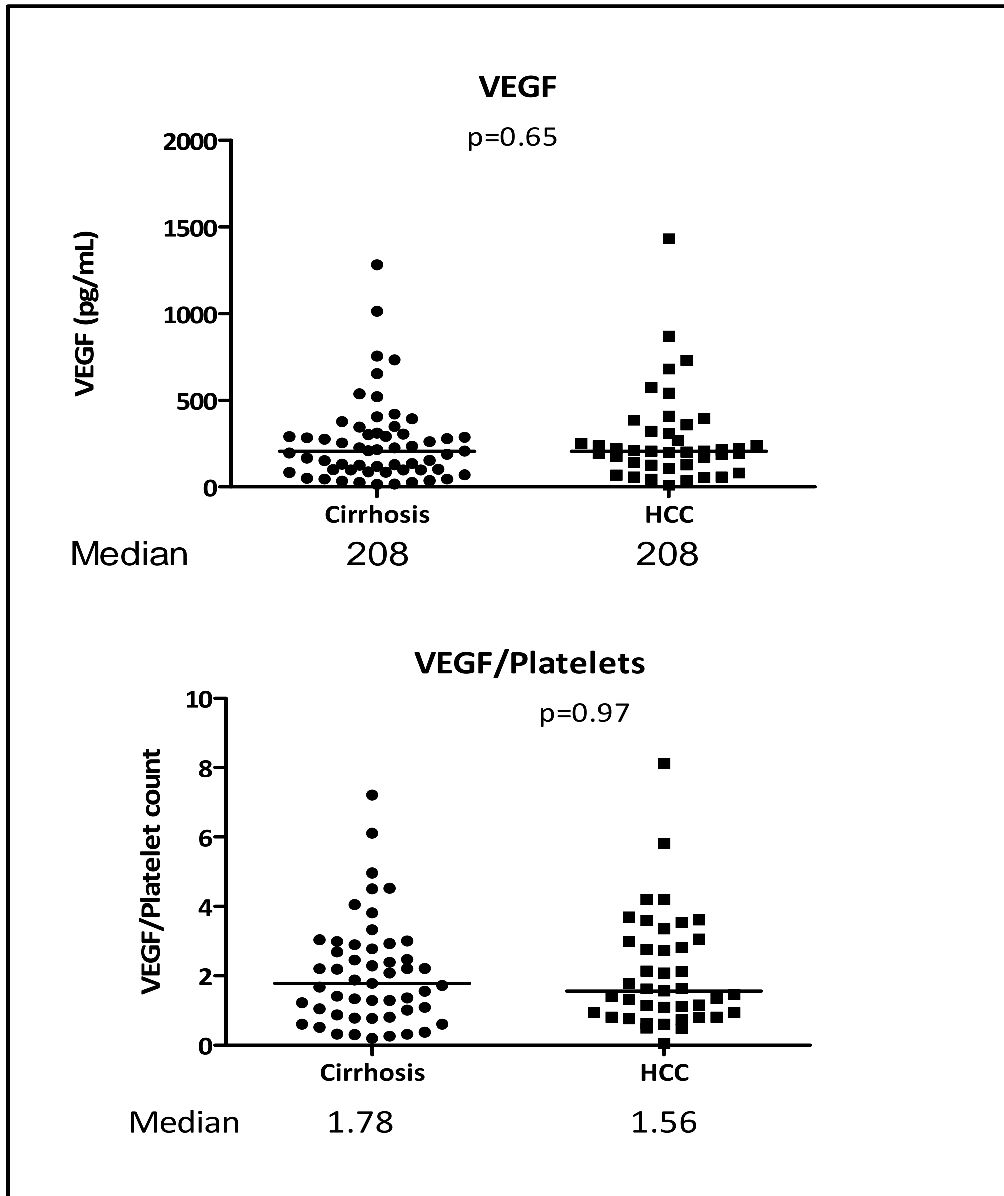


Figure 4.3 VEGF versus VEGF/Platelet number



In Figure 4.3 VEGF levels from patients with HCC and those with cirrhosis alone were compared using two methods, firstly VEGF values alone and secondly VEGF divided by platelet count as recommended by Poon et al (301). Using neither method is there a statistically significant different between the two sample groups**.**

4.8.3 Association between serum cytokines and clinical parameters

Cytokine levels were not affected by age or sex. Interestingly both Ang-2 and VEGF were positively correlated with a raised AFP and increasing tumour size over 8cm but not with the number of tumours (see Table 4.6). Patients with Child Pugh class B / C disease had higher Ang-2 levels although the difference was small in absolute terms. By contrast the level of Ang-1 was not correlated with any of the factors measured. The level of erythropoietin and bFGF were both correlated with aetiology such that those with non-viral aetiology had higher serum Epo and bFGF. bFGF was also correlated with increasing size of the dominant lesion. Whereas serum VEGF was not statistically higher in patients with HCC and a viral aetiology, using Serum VEGF / Platelet count this did reach significance.

For patients with cirrhosis Ang-2 was elevated in those with Child-Pugh stage B/C while Ang-1 was reduced resulting in a highly significant difference in Ang-2/ Ang-1 ratio between the two groups (see Table 4.6).

Table 4.6 Association between cytokines and clinical parameters
:in HCC (A) and cirrhosis (B)
(statistically significant associations are in bold).

A)

	No in brackets	**Ang-1 pg/ml**	**Ang-2 pg/ml**	**Ang1/2 ratio**	**VEGF pg/ml**	**VEGF/ plts**	**Epo MIU/ml**	**bFGF pg/ml**
Age	<60 (20) >60 (25)	14938 20340 P= 0.1	6584 6628 P=0.9	0.41 0.46 P = 0.8	192 217 P=0.1	1.39 1.59 P=0.41	11.9 12.9 P=0.7	2 1.9 P=0.6
Sex	M (37) F (8)	18600 16413 P= 0.5	6641 6574 P= 0.6	0.43 0.42 P=0.98	217 185 P=0.09	1.56 1.43 P=0.35	11.9 14.5 P=0.7	2.2 0.7 P=0.08
Cause	Viral (29) Other (16)	15575 21725 P=0.1	6645 5873 P=0.5	0.44 0.24 P=0.1	205 223 P=0.6	1.56 1.87 P=0.88	**10.2** **16.4** **P=0.005**	**1.4** **6.3** **P=0.02**
αFP µg/ml	<20.6 (23) >20.6 (22)	18295 17870 P=0.86	**4638** **9863** **P=0.0002**	**0.28** **0.59** **P=0.0008**	**133** **247** **P=0.004**	**1.11** **2.42** **P=0.003**	11.1 15 P=0.09	2 2.1 P=0.76
Child-Pugh	A (29) B/C (17)	18850 14418 P=0.31	**5365** **12799** **P=0.002**	**0.28** **0.58** **P=0.002**	194 246 P=0.13	1.4 2.73 P=0.16	12.1 13 P=0.4	2.4 1.9 P=0.6
Size largest nodule (mm)	≤ 3 (18) 3-8 (18) ≥ 8 (9)	16483 17740 28830 P=0.1	**6655** **4814** **12890** **P=0.008**	0.53 0.28 0.44 P=0.07	**211** **192** **391** **P=0.02**	2.43 1.16 2.32 P=0.15	10.6 13.1 16 P=0.4	**1.2** **2.1** **5.3** **P=0.03**
No of nodule	1 (19) 2-5 (18) ≥ 5 (8)	15575 17565 26230 P=0.3	6123 6781 4568 P=0.3	0.38 0.57 0.32 P=0.09	217 208 200 P=0.9	1.77 1.56 1.76 P=0.84	14.4 12.5 10.1 P=0.5	2.7 1.6 1.9 P=0.7

B)

	No in brackets	**Ang-1 pg/ml**	**Ang-2 pg/ml**	**Ang1/2 ratio**	**VEGF pg/ml**	**VEGF/ plts**	**Epo MIU/ml**	**bFGF pg/ml**
Age	<60 (33) >60 (18)	16541 17450 P=0.14	6997 4472 P=0.55	0.49 0.26 P=0.13	208 217 P=0.99	1.78 1.71 P=0.4	18.46 14.66 P=0.94	2.92 3.47 P=0.35
Sex	M (34) F (17)	17150 15630 P=0.73	6677 4720 P=0.33	0.33 0.39 P=0.92	226 152 P=0.23	1.93 1.55 P=0.79	13.38 21.22 P=0.12	3 4.91 P=0.17
Aetiology	Viral (26) Other (25)	14450 18450 P=0.07	6209 4990 P=0.68	0.46 0.33 P=0.36	232 135 P=0.17	**2.58** **1.29** **P=0.003**	13.56 14.66 P=0.92	3.12 3.47 P=0.72
αFP μg/ml	< 4 (29) > 4 (18)	16250 16833 P=0.61	**4720** **9045** **P=0.02**	0.33 0.56 P=0.35	168 245 P=0.16	1.41 2.34 P=0.23	17 10.86 P=0.38	3.11 2.47 P=0.74
Child-Pugh	A (34) B/C (17)	17450 15487 P=0.27	**4077** **10014** **P=0.0001**	**0.26** **0.68** **P=0.03**	232 132 P=0.08	1.98 1.55 P=0.32	15.39 13.93 P=0.93	3.3 3.3 P=0.59
Platelet count	< 140 (32) > 140 (19)	**13153** **32650** **P<0.0001**	**7146** **3802** **P=0.02**	**0.56** **0.11** **P<0.0001**	**160** **303** **P=0.003**	2.14 1.36 P=0.23	15.24 14.66 P=0.34	2.75 4.08 P=4.08

4.8.4 Association with prognosis

The median follow-up for patients with HCC was 574 days (range 13 to 1344 days) with no patients lost to follow up. Survival curves were generated for each cytokine dividing the population into two groups; those above, and those below the median value for the whole population (see Figure 4.4).

Patients with angiopoietin-2 levels above 6628pg/ml had a significantly worse median overall survival of 459 days compared to 923 days for those below. Similarly for patients with VEGF levels above 208pg/ml median survival was only

293 days compared with 923 days for those below. Ang-1, bFGF and Epo levels had no prognostic significance.

Univariate analysis (Table 4.7) confirmed that known prognostic factors for HCC, namely liver function markers (bilirubin and ascites), and tumour factors (AFP and number and size of nodules) correlated with adverse prognosis. Of the cytokines measured only an elevated Ang-2 and VEGF were associated with a poorer survival.

Applying multivariate analysis only AFP, bilirubin and Ang-2 remained significant. Among these factors Ang-2 was the most discriminating in terms of prognosis with a hazard ratio of 5.13 for every 10k unit rise in serum concentration.

Figure 4.4 Survival Curves (Kaplan-Meier)

Comparing the 50% of patients below the median value to the 50% above.

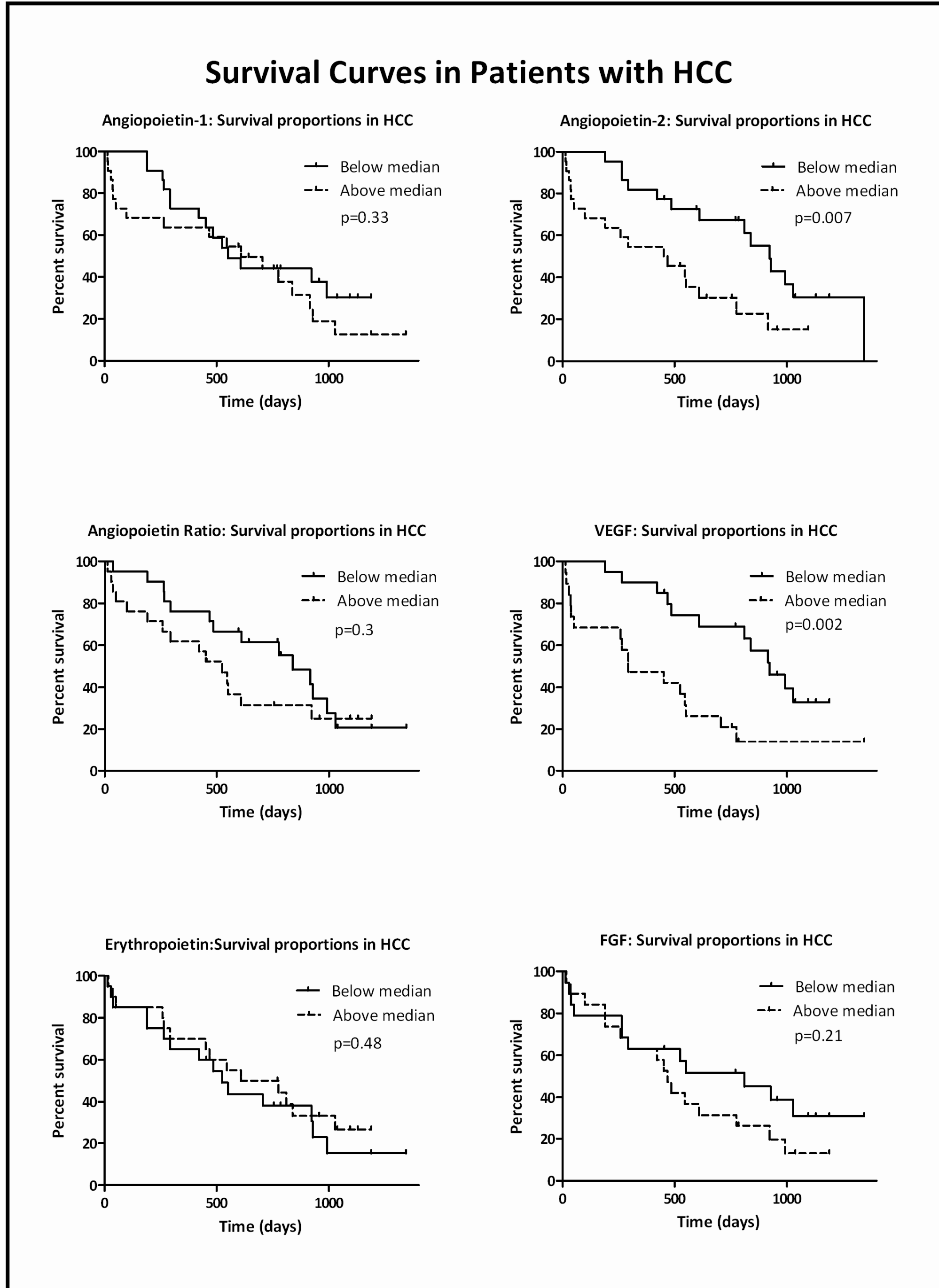


Table 4.7 Univariate (A) and multivariate (B) analysis

Showing relationship between baseline variables and survival outcome. All variables are analysed as continuous variables other than sex, ascites, and alcohol and viral aetiology which are categorical.

A)

Variable	HR	95% CI		p value	N
Male	2.12	0.47	9.70	0.331	45
Age	1.00	0.95	1.05	0.852	45
Albumin	0.85	0.76	0.94	**0.001**	45
Bilirubin	1.05	1.02	1.09	**0.002**	45
INR (per 10 units)	1.29	0.93	1.80	0.127	44
Number of nodules	1.11	1.04	1.19	**0.003**	45
Max. diameter of tumour (cm)	1.21	1.06	1.38	**0.005**	45
Log (AFP)	1.33	1.11	1.60	**0.002**	45
Ascites	5.52	1.90	16.07	**0.002**	45
Alcohol	0.93	0.25	3.37	0.907	44
Viral	0.89	0.32	2.52	0.829	45
Ang-1 (per 10k units)	1.17	0.71	1.90	0.539	44
Ang-2(per 10k units)	6.78	2.87	16.02	**<0.001**	44
VEGF (per 10 units)	1.03	1.02	1.05	**<0.001**	42
Erythropoietin	1.01	0.97	1.04	0.619	41
BFGF	1.04	0.90	1.22	0.577	38

B

Variable	HR	95% CI		p value	N
Ang-2(per 10k units)	5.13	1.84	14.29	**0.002**	44
Log (AFP)	1.30	1.05	1.61	**0.015**	45
Bilirubin	1.04	1.00	1.09	**0.036**	45

4.8.5 Cytokine changes with embolisation

In order to determine if acute tumour hypoxia caused a rise in serum cytokine levels blood was collected before and at various time points up to 21 days following transarterial embolisation (see Table 4.8). Not all patients provided samples at each time point and therefore the baseline values shown are those corresponding to each time point. In order to control for the acute effects of the angiographic procedure associated with TACE, cytokine levels in patients undergoing cardiac angiography were also measured.

For Ang-2 there was a small increase at 24 hours but no significant difference at any other time after TACE compared to baseline. VEGF levels rose to a peak at day 15 when they were twice that at baseline. Serum Epo almost doubled at 24 hours post TACE and remained elevated at 7 days returning to baseline levels at 21 days. An example of a patient having 3 separate embolisations demonstrating Epo rise with each one is shown in Figure 4.5. bFGF did not change significantly at any point.

In the control cardiac angiography group, there was a small but significant increase in Epo at 24 hours but the change was only 21% compared to the 76% rise seen in HCC patients at the same time point suggesting the additional hypoxic event in TACE causes a greater rise in Erythropoietin.

Table 4.8: Cytokine levels at baseline (Bl) and following TACE.
For patients with HCC (A) or coronary angiography in control cardiac patients (B) (statistically significant changes are in bold).

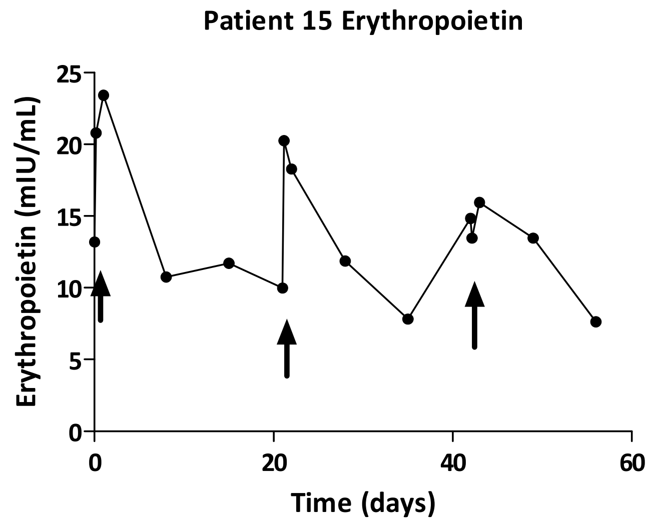
A

Times	**BL**	**4 hr**	**BL**	**24 Hr**	**BL**	**8 days**	**BL**	**15 days**	**BL**	**21 days**
	N = 8		N = 25		N = 7		N = 7		N = 9	
Ang-1	18420	18720 P=0.62	15500	15413 P=0.87	15565	23733 P=0.31	17565	16865 P=0.31	17350	17275 P=0.91
Ang-2	5123	5813 P=0.55	**6415**	**6517 P=0.03**	6415	6600 P=0.47	6415	6880 P=0.38	6415	6773 P=0.43
VEGF	135	113 P=0.23	190	219 P=0.37	141	197 P=0.13	**133**	**276 P=0.02**	187	235 P=0.19
Epo	11.9	13.3 P=0.62	**12.6**	**22.2 P=0.004**	**11**	**17.2 P=0.04**	11.5	17.1 P=0.08	10.6	11.8 P=0.84
bFGF	0.77	1.13 P=0.055	1.93	2.65 P=0.3	0.7	1.66 P=0.082	0.44	0.87 P=1	1.31	2.01 P=0.92

B

Cardiac patients	**Baseline**	**24hr**
	n=22	n=22
Ang-1	30363	32936 *p=0.38*
Ang 2	3180	2853 *p=0.08*
VEGF	232	244 *p=0.4*
Epo	**5.3**	**6.4** ***p=0.05***
FGF	4.62	4.71 *p=1*

Figure 4.5: Example of Erythropoietin post embolisation.
Arrows represent time of TAE (Trans-arterial embolisation)



4.8.6 ROC Curves

ROC (Receiver Operating Characteristic) Curves plot the sensitivity of a test (or the true positive rate) against 1-specificity (false positive rate) on each axis. This can be used to assess how effective these biomarkers are at distinguishing patients with a condition (HCC or cirrhosis) from those without. The test is used in two settings, to attempt to distinguish patients with HCC from those with cirrhosis alone (Figure 4.6) and to distinguish patients with cirrhosis from normal healthy controls (Figure 4.7).

The first set of curves demonstrates little ability for our selected angiogenic cytokines to differentiate patients with cirrhosis and HCC from those with cirrhosis alone. Only bFGF has a significant p-value (0.033), but with a low AUC value of 0.63. The current standard, α-FP, performs much better with a p-value of < 0.0001 and an AUC of 0. 85.

The second set considers patients with early stage cirrhosis (Child-Pugh A disease) and the healthy control group. Here the angiogenic cytokines seem more effective. As AFP was not measured in the healthy control group it was not possible to compare to that biomarker. Scholz et al considered the combination of Ang-2 with AFP in the detection of HCC and found the combination more effective than when either Ang-2 or AFP were used alone rather than in combination (348). However whether Ang-2 could be useful in the diagnosis of cirrhosis was not discussed.

Figure 4.6: ROC Curves HCC versus Cirrhosis

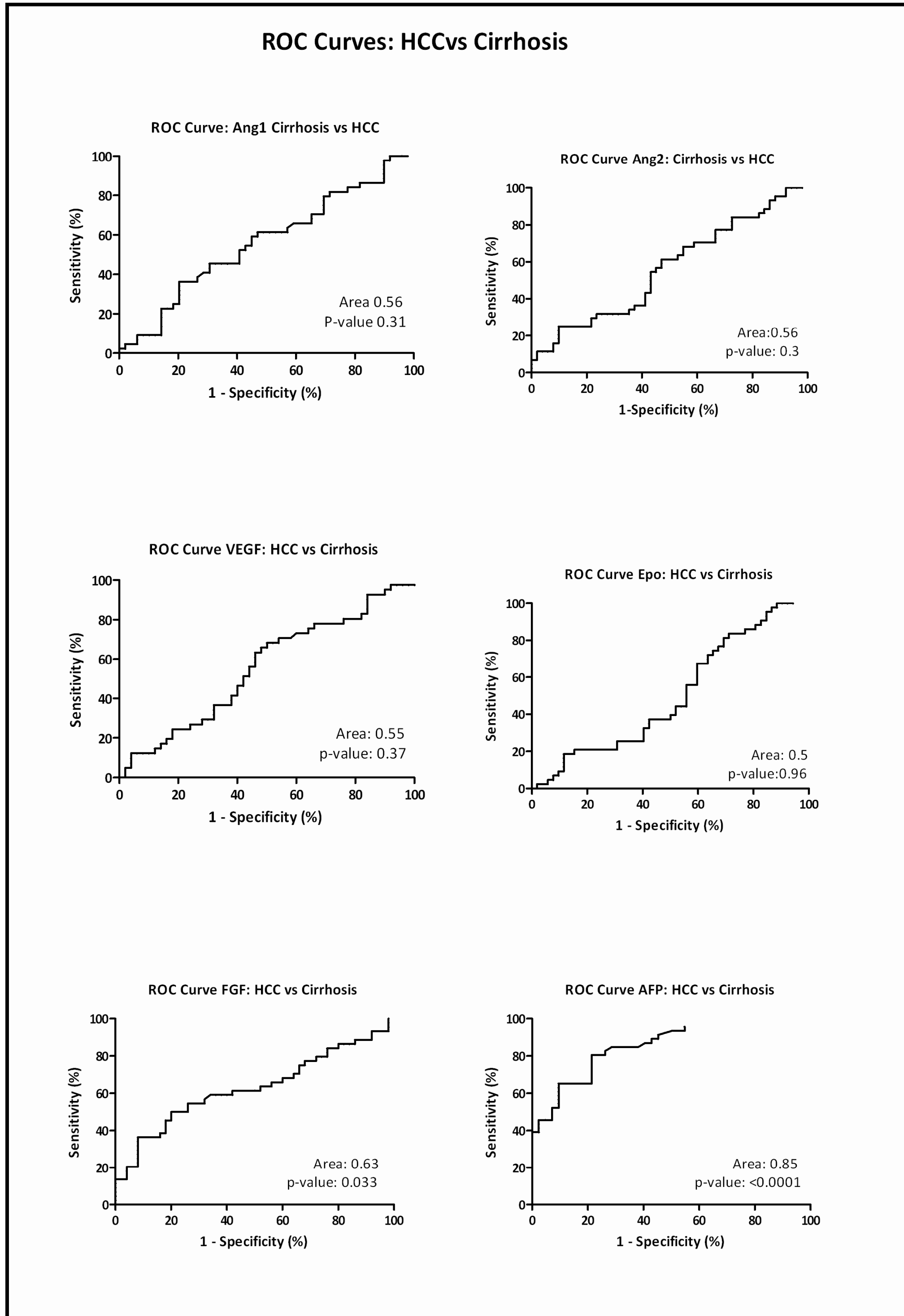
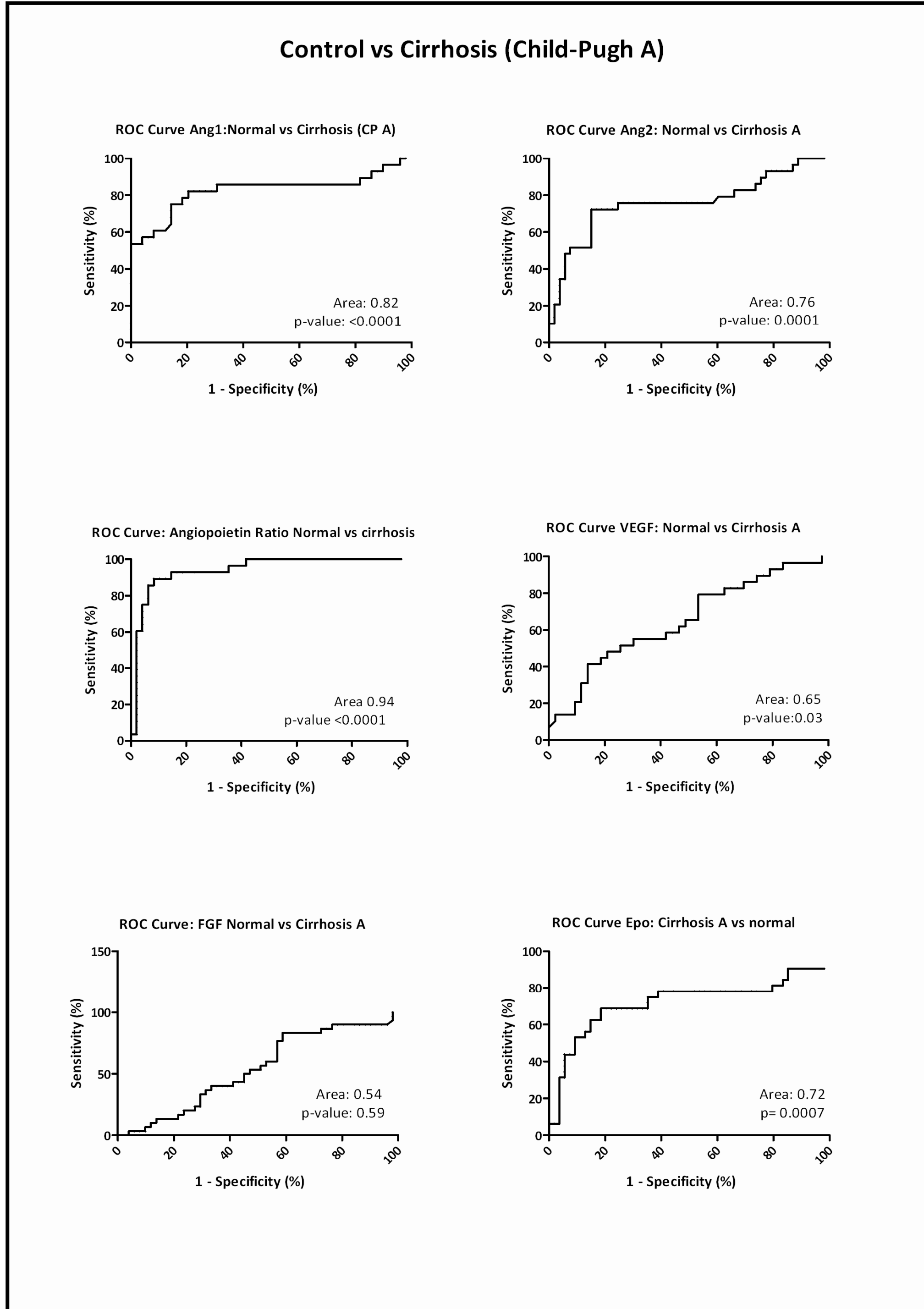


Figure 4.7 : ROC Curves Control vs. Cirrhosis (Child-Pugh A only)



4.9 Conclusions

The aim of this study was to identify a blood based biomarker for the acute hypoxia induced by VDA's that could compliment the more expensive and technically demanding imaging assessments. We chose to focus on angiogenic cytokines that are up-regulated by hypoxia and used the model of TAE for HCC to explore the feasibility of this approach. In performing this analysis we also discovered significant differences between certain cytokines in patients with cirrhosis and HCC compared to healthy controls which may have important clinical applications.

Ang-2 appears to be a strong and independent prognostic factor for survival in hepatocellular cancer, with the risk of dying increasing fivefold for every 10,000 units increase in Ang-2. The ratio between Ang-2 and its antagonist, Ang-1, is increased in patients with cirrhosis and HCC reflecting a pro-angiogenic milieu although there was no significant difference between HCC and cirrhosis for either Ang-1 or Ang-2. Higher levels of Ang-2 were associated with more severe liver disease, α-FP and an increased size of the largest nodule. Other authors have not demonstrated relationships between the angiopoietins and poor prognostic clinico-pathological markers. In addition the failure of Ang-2 to rise post-embolisation, even with tumour necrosis occurring suggests it may be secreted by the surrounding liver rather than tumour. The lack of a rise post-embolisation suggests it would be an ineffective biomarker for the anti-vascular agents.

Serum Ang-1 was reduced in patients with cirrhosis alone and with HCC compared to healthy controls. Ang-1 was not found to be a prognostic factor and no changes were seen post embolisation. This confirms work from tissue expression studies (334, 399). Most reports from other cancers find equivalent

serum Ang-1 between controls and cancer patients (344, 345) apart from a study in lung cancer where serum Ang-1 was reduced (342). It may be that Ang-1 levels also are affected by the surrounding cirrhotic tissue. Serum Ang-1 is unlikely to represent an effective biomarker.

Previous work has shown an over-expression of VEGF mRNA in HCC (327, 334, 363, 400). The highest expression was found adjacent to areas of necrosis (392) consistent with a response to hypoxia (401). A recent meta-analysis identified eight studies that had explored the prognostic value of serum VEGF in HCC (300) and all but one were performed in the Asian population. In keeping with this analysis, we found that higher levels were significantly associated with a worse prognosis. However unlike Poon et al (364) we found no difference between VEGF levels in HCC patients and healthy controls. It should be noted that the normal range for serum VEGF is wide and a small control cohort may provide misleading results. As predicted serum VEGF did rise post-embolisation secondary to tumour ischaemia. The time-course was relatively slow in this group of patients and only peaked at 15 days. Potentially changes in VEGF could therefore be explored in patients treated with VDAs.

In HCC bFGF is thought to act via the augmentation of VEGF in an autocrine manner (402). Previous work demonstrates both an increased tissue expression of bFGF in HCC (403) and correlation with the MVD (350). All published studies assessing serum bFGF have been done in Asian patents and, while one has shown serum bFGF to be associated with a poor survival (364) two other studies showed no such correlation (360, 365). In agreement with Poon et al (364) we found a significant association between tumour size and serum bFGF but, in keeping with the two other studies we did not find an association with prognosis. bFGF failed to rise post embolisation and would therefore not represent a good biomarker for the anti-vascular agents.

Erythropoietin has been implicated in angiogenesis (404, 405). Previous studies have reported an elevated serum Epo in patients with HCC and considered it a likely explanation for the erythrocytosis sometimes observed (406-408).

Erythropoietin over-expression in HCC correlates with a poorer differentiation and increased microvessel density (389), however the prognostic significance of a raised serum Epo has not been explored. In our study we found that serum Epo was significantly elevated in patients with HCC and cirrhotics but no significant difference was observed between the two groups. Erythropoietin levels were higher in those with a non-viral aetiology but no other association with clinical factors was seen, nor any association between Epo levels and prognosis. Transcription of Epo is regulated by HIF1α in response to hypoxia and it is therefore a potential marker of acute ischemia.

It is of interest that Erythropoietin levels in our study almost doubled in 24 hours after TACE and remained significantly elevated for at least one week. Erythropoietin levels also rose post cardiac angiography but to a lesser extent, which may reflect a lower level of ischaemia in patients having this procedure. The rapid and consistent rise in erythropoietin levels would merit this cytokine being investigated further as a potential biomarker for the anti-vascular agents.

The differing time course of response for VEGF and Erythropoietin to TAE may partly be due to the biology of the two cytokines. Erythropoietin is a systemic hormone with a half life of approximately 6 hours; whilst VEGF is locally acting with a half life in the circulation of 3 minutes. It is likely that additional factors explain the late rise in VEGF rather than acute ischaemia. I have investigated VEGF using both serum VEGF and also serum VEGF/platelet count as advocated by Poon et al (301). In this study both methods appear to give equivalent results.

Other biomarkers for VDA's have been considered. These include markers such as the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) which becomes elevated post DMXAA administration. This rise is thought to be via platelet aggregation secondary to vascular damage with secretion of the vasoactive amine serotonin. Both Ang-1 and Ang-2 act via the Tie-2 receptor; located mainly on endothelial cells and haematopoietic cells, including a subset of monocytes that appear important in tumour angiogenesis. In addition both erythropoietin and VEGF are thought to promote the recruitment of endothelial progenitor cells (EPC's) from the bone marrow that may promote angiogenesis. These will be considered further in the next chapter.

In addition we have demonstrated that angiogenic cytokines are elevated in cirrhosis. Whilst the VEGF/Platelet count was higher in patients with a viral aetiology and may be related to inflammation, the other cytokines showed no correlation with aetiology and are more likely associated with cirrhosis itself. ROC curves show a statistically significant difference between patients with early stage cirrhosis (Child-Pugh A) and healthy controls for both angiopoietins and erythropoietin. These findings should be considered for further research. Angiogenic cytokines such as VEGF and Angiopoietin-2 have already been implicated in the development of cirrhosis (348) although the mechanism remains obscure. Further investigation in their role in the diagnosis and as a marker of prognosis appears warranted.

Chapter Five: Tie-2 monocytes and Endothelial Progenitor Cells as biomarkers for Vascular Disruptive Agents.

In the Introduction I considered two cell types that might act as potential biomarkers for vascular shut-down induced physically by embolisation, or biologically by the VDA's. These are the group of monocytes that express the Tie-2 receptor and endothelial progenitor cells. I shall briefly recap on their properties below before reporting the clinical trial data.

5.1 Tie-2 Expressing Monocytes

Approximately 20% of circulating monocytes are thought to express the Tie-2 Receptor (158). In addition these cells can be identified by co-expression of the classical monocyte markers of CD14, CD16 and CD11b and the lack of endothelial cell markers (161) such as VEGFR2. In mouse models they are known to localize to sites of angiogenesis and human tumours (159). In humans they localize to the peri-vascular space and areas of hypoxia; their distribution may reflect that of Angiopoietin-2, released secondary to that hypoxia (158).

Physiologically TEM's are thought to be involved in tissue growth and regeneration (163). In malignancy they are thought to be pro-angiogenic and their ablation results in a reduction in tumour growth and angiogenesis (159). Tie-2 monocytes respond to Angiopoietin-2 or hypoxia by suppressing secretion of IL-12 and TNF-α (165). It is hypothesized in our study that they are recruited from the circulation by the hypoxia generated by embolisation. Recruited to tumour they would aid angiogenesis and augment tumour growth. They may therefore represent a potential biomarker for vascular shut-down.

5.2 Endothelial Progenitor Cells

Endothelial Progenitor cells are thought to be bone-marrow derived cells that retain the ability to migrate, form primitive tubes and adhere to substratum as well as possessing a high proliferation rate (169). They are thought to be recruited to areas of neo-vascularisation guided by the angiogenic cytokines where they differentiate into endothelial cells and aid angiogenesis (170). There is no standard way to define an EPC. They are thought to express the markers VEGFR2, CD34, CD31, CD133 and CD146 (176) although these markers may encompass a number of cellular sub-groups with differing properties.

Numbers isolated from the circulation depends on the technique used to isolate them. Flow cytometry values are typically 70-210 cells/mL of blood (using CD34/133/VEGFR2) (179). In malignancy EPC's are thought to aid angiogenesis and have been associated with tumour progression. Their recruitment from bone-marrow is thought to be secondary to hypoxia via the release of HIF1-α (206) with blood levels rising within 24 hours. The level of incorporation into the tumour-associated vasculature and the effect this has remains controversial and may differ depending on stage, grade, animal model and tumour type (189). EPC's have been investigated previously as biomarkers for anti-angiogenic agents (178) and for the VDA's in a mouse model (202) where levels rose approximately 4 hours after administration.

In our hypothesis trans-arterial embolisation would generate ischaemic necrosis with hypoxia. Subsequently EPC's would be recruited from the bone marrow. Levels would rise in the blood where they could be recorded via flow cytometry with the potential of acting as a biomarker for vascular shut-down.

With this aim I investigated levels of Tie-2 expressing monocytes and Endothelial Progenitor Cells in patients with hepatocellular cancer, both pre and post TAE. The results are presented below.

5.3 Hypothesis

Our hypothesis was that the acute hypoxia induced by embolisation or by VDAs would lead to an increase in angiogenic cytokines. An increase in angiopoietin-2 may cause Tie-2 monocytes to be recruited into tumour from the bone marrow and promote further angiogenesis. We attempted a preliminary study to evaluate the feasibility of assessing the level of Tie-2 monocytes in the peripheral blood as a biomarker that could in future be used to assess the VDA agents.

In addition, as endothelial progenitor cells are also recruited to tumours under the influence of angiogenic cytokines and may themselves enhance angiogenesis, we investigated EPCs as a biomarker in HCC. As no standard methodology exists for EPCs three separate methods were explored.

5.4 Methods

I analysed Tie-2 positive monocytes in peripheral blood in a group of patients with hepatocellular cancer who were treated with TAE. The technique was evaluated for feasibility with 8 patients whose blood was selected anonymously who were receiving chemotherapy*. A control group of 11 was selected; 6 of these were healthy controls and 5 were undergoing coronary angiography.

* Blood samples collected from the chemotherapy clinic were reviewed in the haematology laboratory as per standard clinical practice. The supervising clinical

chemist removed all identifying data from the samples including name, sex, hospital number and date of birth after completing their standard clinical investigations. I then received the remaining blood which would otherwise have been discarded with no identifying data present.

5.4.1 Sample collection and analysis

5.4.1.1 Tie-2 monocytes

I analysed the whole blood concentration of Tie-2 positive monocytes using flow cytometry. A 'lyse, no-wash' protocol was followed due to the likely low frequency of positive cells in the blood. The alternative method with density gradient separation and washing has been found to be poorly reproducible (409).

Each sample was prepared and analysed in triplicate. 4mls of EDTA preserved blood was collected and stored until ready to analyse. Flow cytometry was performed within 4 hours of blood collection. 200μl samples were aliquoted into 3 test-tubes (polypropylene) and mixed with 5μl of each fluorochrome-conjugated antibody. The combinations of monoclonal antibodies are documented below. Antibodies were stored at 2-8˚C in the dark.

The fluorochrome-conjugated antibodies used were:

1. PE (Phycoerythrin) stain against CD11b (BD Biosciences code 3331412)
2. FITC (Fluorescein Isothiocyanate) stain against CD14 (BD Biosciences code 345784)
3. PerCp (Peridinin Chlorophyll) stain against CD45 (BD Biosciences code 345809)
4. APC (Allophycocyanin) stain against Tie-2 (R&D Systems code FAB3131A)

The combination was then mixed using a laboratory vortex shaker and left at room temperature for 15 minutes in the dark. The red cells were lysed using Easylyse™ (Dako) at a concentration of 1 in 20 with distilled water. The sample was then centrifuged at 200G for 5 minutes with brake on. The supernatant liquid was discarded and the pellet containing the cells of interest was re-suspended in 1ml of buffered isotonic salt solution (PBS) for flow cytometry. The samples were analysed immediately by flow cytometry. Initially the samples were analysed using the FACSCalibur™ model (BD Science) with WinMDI software or the FACS Aria™ (BD) Cellsorter. Analysis was done blinded to the patient identity or time point of the sample to reduce bias.

The strategy to detect Tie-2 positive monocytes includes the use of CD45 as an electronic threshold signal to allow the exclusion of erythrocyte debris from the acquisition. Monocytes are medium sized cells and therefore have a medium forward scatter. They also have a medium granularity with a medium side scatter. They can be identified on a plot of those two variables and an "acquisition gate" set around them. These selected cells are confirmed to carry the monocyte markers CD14 and CD11b.

As Tie-2 positive monocytes are relatively rare events in peripheral blood, leukocytes were continually acquired until > 10,000 events had been collected that met criteria for both forward and side scatter. The entire data file was approximately 250,000 leukocytes.

5.4.1.2 Gating strategy for Tie-2 monocytes

The gating strategy is shown pictorially in Figure 5.2. Initially forward scatter and side scatter was used to distinguish granulocytes, lymphocytes and monocytes. A FITC stain for CD14 distinguishes the monocytes which are gated in R1. To further confirm the cells are truly monocytes they were gated with

CD11b and then back gated to forward and side scatter graphs (not pictured). Finally the monocyte population was gated against Tie-2 APC. The distinction between positive and negative was judged on where the population clusters of Tie-2 negative cells appear to end. If this was indeterminate back- gating could be performed to judge from the negative populations of lymphocytes or granulocytes.

5.4.1.3 Statistical analysis

The data was analyzed using WinMDI (Version 28). The data was then analysed further with GraphPad Prism 5 software (Software, San Diego, CA). Tie-2 monocyte values were expressed as median values and compared using the Mann-Whitney U test. The Kaplan-Meier method was used to estimate overall survival curves and survival curves were compared using the log-rank test.

5.4.2 Endothelial Progenitor Cells

Blood was collected and stored using the same protocol described above with the Tie-2 positive monocytes. A 'lyse, no-wash' procedure was used for flow cytometry as explained above. Again 200 μl aliquots of blood was incubated at room temperature for 15 minutes with the following combinations of 5 μl each of these fluorochrome-conjugated monoclonal antibodies. The procedure followed the same method from then as with the Tie-2 expressing monocytes. Again the analysis was completed blinded to the identity of the sample and its time order to reduce bias.

A number of different combinations of fluorochrome-conjugated antibodies were considered for evaluation of EPCs as detailed below:

Method One

FITC stain against CD34 (BD Biosciences Code no 345804)

PE stain against CD133 (Miltenyi Biotech Code no 130-080-801)

PerCP stain against CD45 (BD Biosciences Code no 345809)

APC stain against VEGFR2 (R&D Systems Code no MAB3572)

Method Two (Based on a protocol by Dulic-Sills et al (444))

FITC stain against CD2/CD13/ CD22

PE stain for CD133 (Miltenyi Biotech Code no 130-080-801)

PerCP stain against CD45 (BD Biosciences Code no 345809)

APC stain against VEGFR2 (R&D Systems Code no MAB3572)

Method Three

FITC stain against CD146 (Serotec Code no MCA2141)

PE stain against CD133 (Miltenyi Biotech Code no 130-080-801)

PerCP stain against CD45 (BD Biosciences Code no 345809)

APC stain against CD34 (R&D Systems Code no MAB3572)

CD45 enabled exclusion of erythrocyte debris and ensured that only leukocytes were acquired for analysis. Initially it was planned to set an "acquisition gate" around cells with a low forward and low side angle scatter (the physical characteristics of EPCs) until 10^6 in that gate had been acquired. In practice acquisition was continued until sample depletion. This required the acquisition of large data files containing 1-3 x 10^6 leukocytes was anticipated.

Due to the rarity expected for endothelial progenitor cells (0.01-0.0001% of mononuclear cells (410), a file of 10^6 of these cells would contain between 10-1000 EPCs (or 70-210 cells/mL (179)). Time would be included as a parameter during acquisition to reduce the distortion of electronic noise affecting the results. Electronic noise can occur either as multiple events at the same time

period or with a regular periodicity rather than rare cell events which occur randomly throughout the period of event acquisition (409). The flow cytometer was cleaned and washed prior to analysis to remove any residual cells or particles.

Using method three the gating strategy was altered after the first 10 patients to try and improve the selection of potential EPC events. In this gating strategy, 200μl of blood was used and lysed carefully. An initial 10000 events were assessed and an individual gate was devised to cover the monocytic population. Events were then collected in this gate until sample depletion. All events outside the gate were discarded and not recorded. This has the advantage of reducing the size of the computer files to assess data which were becoming unwieldy.

5.4.2.1 Statistical Analysis

Rare event analysis is considered to follow a normal distribution. The coefficient of variance can then be determined by cv =100/√N where N is the number of positive events analysed. Therefore if a sample contained 100 true events the cv should be 10% which was felt to be acceptable for this pilot study (409).

The data was analyzed using WinMDI (Version 28) and GraphPad Prism 5 software (Software, San Diego, CA) as described above.

5.4.2.2 Gating strategies

The first gating strategy is shown in Figure 5.7 (179). Initially the physical characteristics of forward scatter and side scatter were used to determine mononuclear and lymphocytic cells. The second gate was around cells that were CD45 negative or dim. The second and third gates isolated cells that were

VEGFR2, CD133 and CD34 positive. These cells were then considered to be endothelial progenitor cells.

The second strategy was developed by Dulic-Sills et al (411) and is shown in Figure 5.8. Here the mononuclear-lymphocyte group is isolated in the same way as above. Then a combination of CD2, CD13 and CD33 was used to exclude cells of haematopoietic origin. Positivity of CD133 and VEGFR2 should then define endothelial progenitor cells.

A third strategy shown in Figure 5.9 (175) was to start again by defining mononuclear and lymphocytic cells followed by those cells that are CD45 low. Then a combination of CD146, CD133 and VEGFR2 positivity could define endothelial progenitor cells.

5.5 Results

5.5.1 Patient Characteristics for Tie-2 monocytes

Between October 2006 and April 2008 blood was collected from 29 patients with HCC from the oncology clinics at the Royal Free Hospital. The blood was collected from the same patient group studied in Chapter Four. In addition anonymised serum was collected from 8 patients undergoing chemotherapy for cancer and 11 healthy controls, including staff volunteers. Inclusion criteria for the patients with HCC were discussed in the previous chapter. The study was approved by the Local Ethics Committee and written informed consent was obtained for all subjects.

Patient characteristics are shown below in Table 5.1. The majority of patients with HCC were male, had a background of hepatitis C infection and Child-Pugh class A disease. Most had multifocal disease confined to the liver with a maximum tumour dimension of 20-50mm. Of these 23 patients had further blood samples taken post-embolisation.

Table 5.1 Patient Characteristics

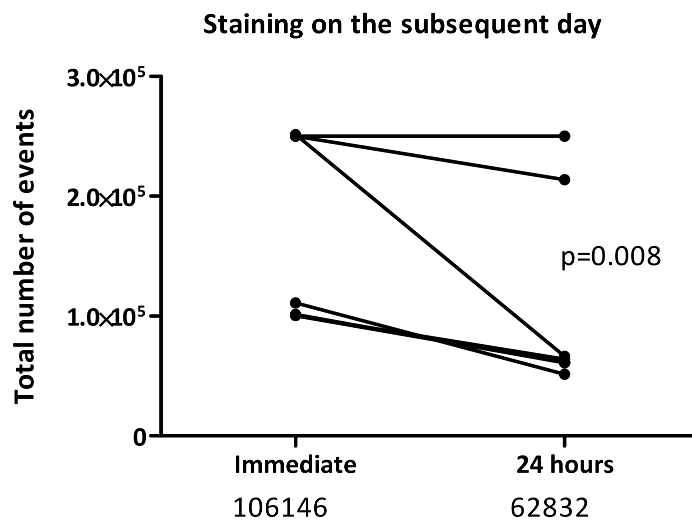
	CHARACTERISTICS	PATIENTS WITH HCC
Age (years)	Mean (range)	60 (38-77)
Sex	Male Female	20 7
Aetiology	HCV / HBV Alcohol Cryptogenic Autoimmune / NASH Haemachromatosis Portal vein thrombosis	13 / 3 6 3 1 / 2 1 1
Albumin (g/dL)	Median (range)	15 (6-56)
Bilirubin (μmol/L)	Median (range)	39 (28-48)
Serum αFP (μg/L)	Median (range)	20.6 (2-88,182)
Platelets (x10^9/L)	Median (range)	108 (43-349)
Child-Pugh score	A B	18 9
Maximum diameter (1 patient had no scan available	Median(range) mm ≤ 2cm (no patients) 2-5cm > 5cm	35 (14-140) 2 15 10
Distribution	Unifocal Multifocal	10 17
Therapy	Embolisation Chemoembolisation Palliative care	18 6 3

5.5.2 Assessing storage of samples

In order to determine the reliability of analysing samples 24 hours after blood was collected we compared the number of events recorded when samples were analysed immediately compared to storing the blood at 4˚C overnight and analysing the following day. As demonstrated in Figure 5.1 there was a significant reduction in event count at 24 hours and for subsequent experiments analysis was performed immediately.

Figure 5.1 Assessing re-staining on the subsequent day

Total number of cellular events counted by the flow cytometer when analysed immediately and at 24 hours after collection.

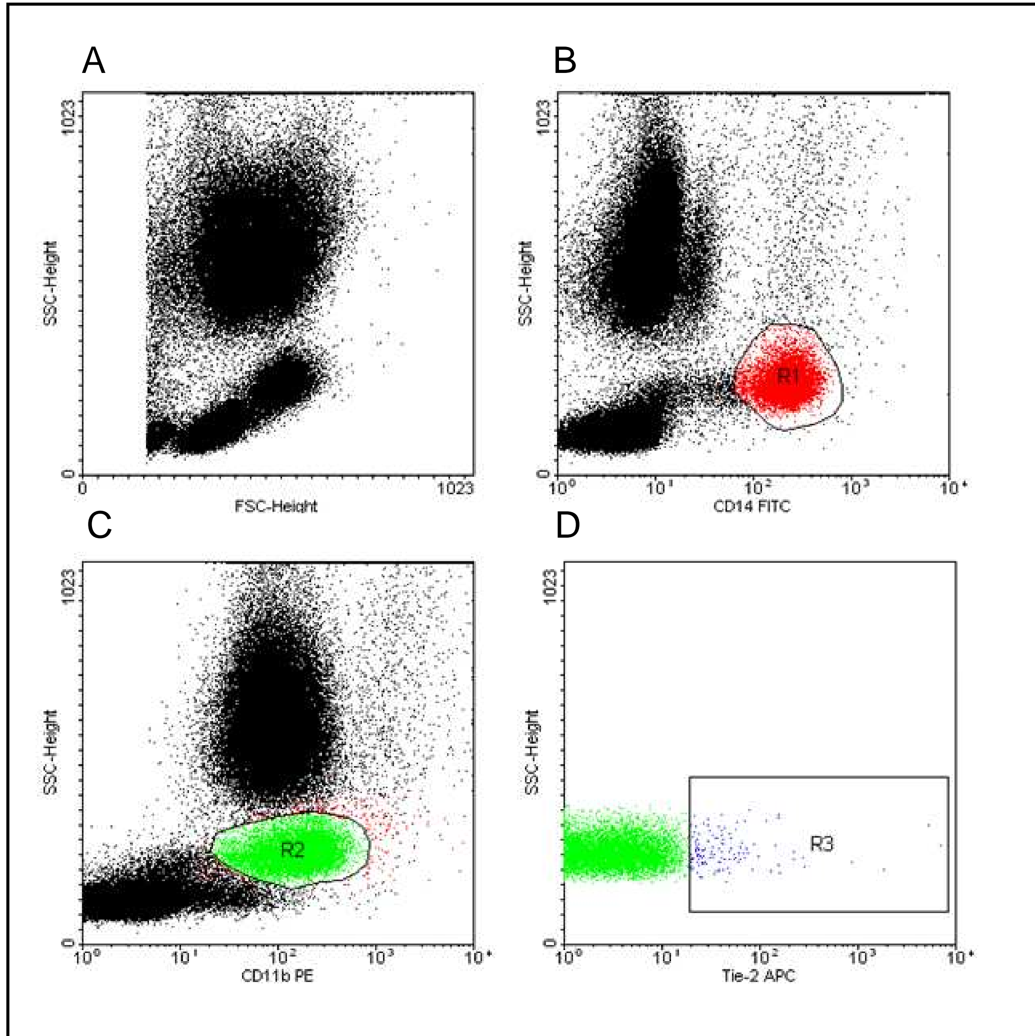


5.5.3 Baseline values for Tie-2 monocytes

The gating strategy for Tie-2 monocytes is demonstrated in Figure 5.2. It demonstrates the presence of cells carrying the monocyte markers CD14 and CD11b as well as Tie-2 consistent with Tie-2 monocytes.

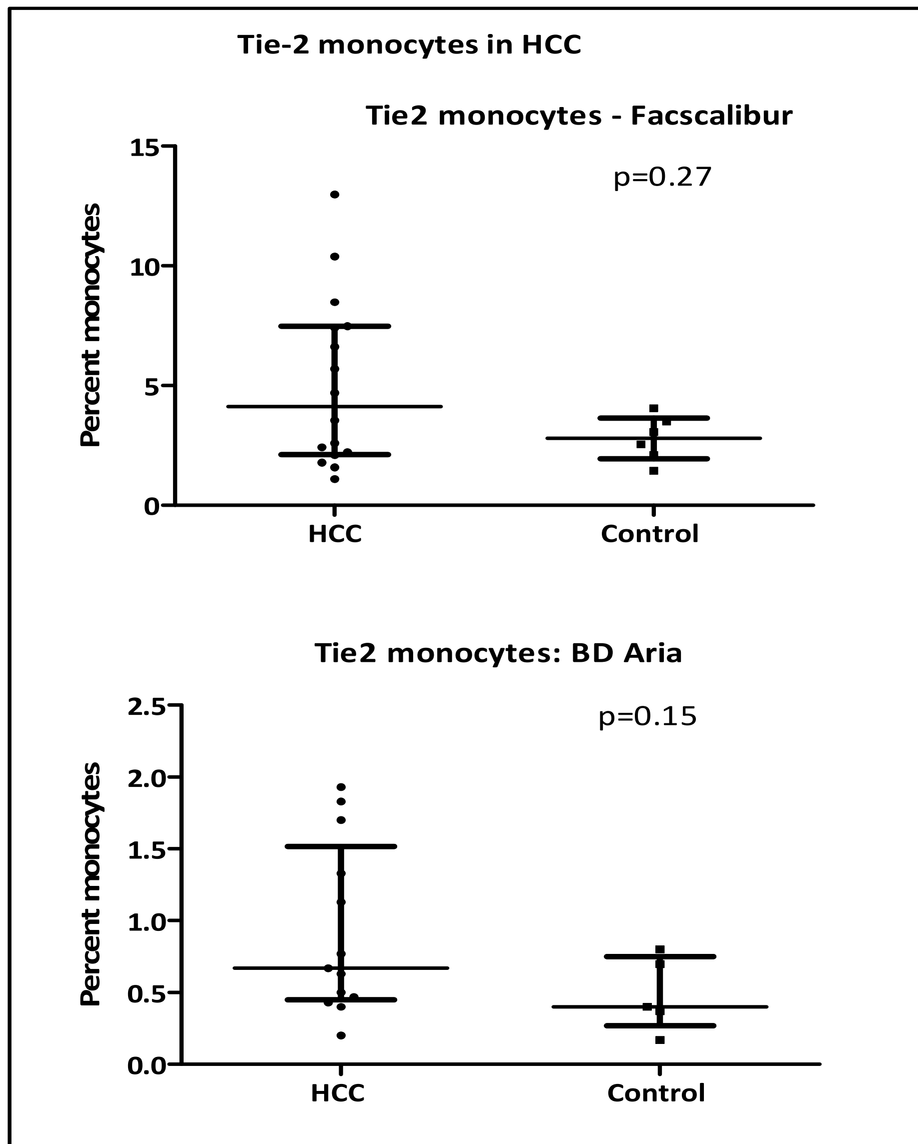
Figure 5.2 Gating strategy for Tie-2 monocytes

A) Gate of side scatter versus forward scatter to delineate cell size and morphology; B) Gate of side scatter versus CD14 expression, R1 demonstrates the monocyte population; C) Gate side scatter versus CD11b expression to confirm monocyte population; D) Gate of side scatter versus Tie-2 showing monocyte population only, R3 shows those positive for Tie-2 expression.



Baseline values for Tie-2 monocytes as a percentage of total monocytes are provided in Figure 5.3. The values were obtained on two separate flow cytometers as a new cytometer superseded the old. The values on the second (the BD FACS Aria) are significantly lower, which corresponded to a new batch of Tie-2 antibody. The values are therefore detailed separately.

Figure 5.3 Baseline values for Tie-2 monocytes



5.5.4 Association between Tie-2 monocytes & clinical parameters

Due to the disparity with the two flow cytometers, only the values from the FACSCalibur are presented here due to a larger sample size. The FACSCalibur data is also closer to previous estimates of the Tie-2 monocyte population in cancer e.g. by Venneri et al (158) who estimated them at 3.3% +/- 1.5% of monocytes.

In this preliminary sample of only 14 patients one clinical parameter reached significance (see Table 5.2). An αFP level of greater than the median of 20.6 ng/ml was associated with an elevated level of Tie-2 circulating monocytes (using the Mann-Whitney U statistical test and median values).

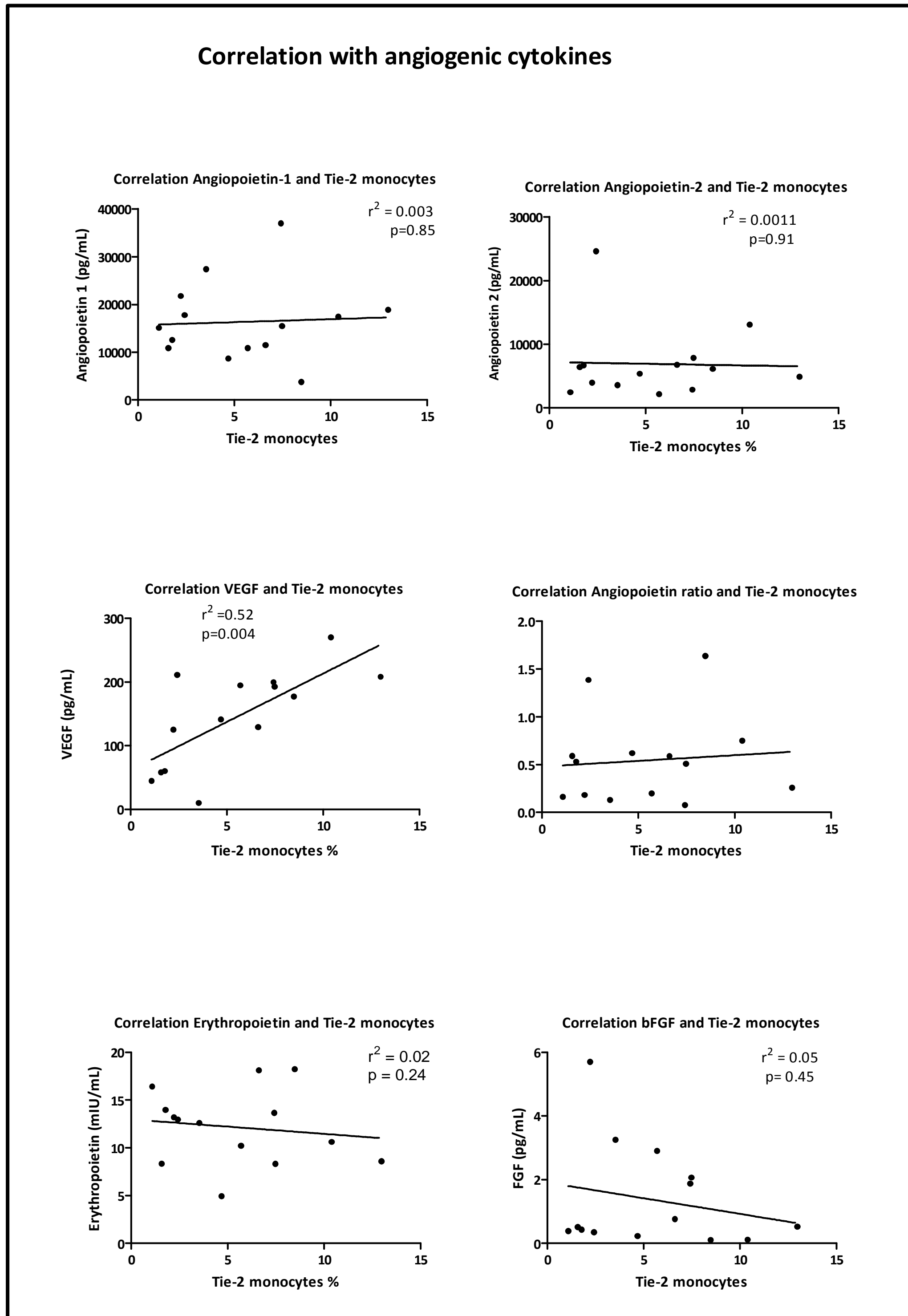
Table 5.2 Tie-2 Monocytes and Clinical Parameters

	CHARACTERISTICS PATIENT NUMBER	TIE-2 MONOCYTES (% MONOCYTE POPULATION) (FACSCALIBUR ALONE)
Age	< 60 years 6 > 60 years 8	3.56 6.62 P=0.27
Sex	Male 7 Female 7	3.56 7.48 P=0.94
Aetiology	Viral 10 Other 4	5.19 5.29 P=0.86
αFP (μg/ml)	< 20.6 8 > 20.6 6	3.54 7.98 P=0.03
Child-Pugh status	A 9 B/C 5	5.49 2.42 P=0.68

5.5.4.1 Associations with angiogenic cytokines

Correlation of the angiogenic cytokines against Tie-2 monocytes is shown in Figure 5.4. Serum VEGF appears to positively correlate against Tie-2 monocytes while angiopoietins 1 & 2 and the angiopoietin ratio show no evidence of any correlation. The significance of the correlation between VEGF and Tie-2 expression in this data-set it remains unclear. The level of correlation is relatively low and the data-set is small. As only 1 of 6 values shows such correlation it may reflect chance alone. It would need to be corroborated in a larger data-set before it could be considered truly significant.

Figure 5.4 Correlation with the angiogenic cytokines



5.5.5 Tie-2 Monocytes changes with embolisation

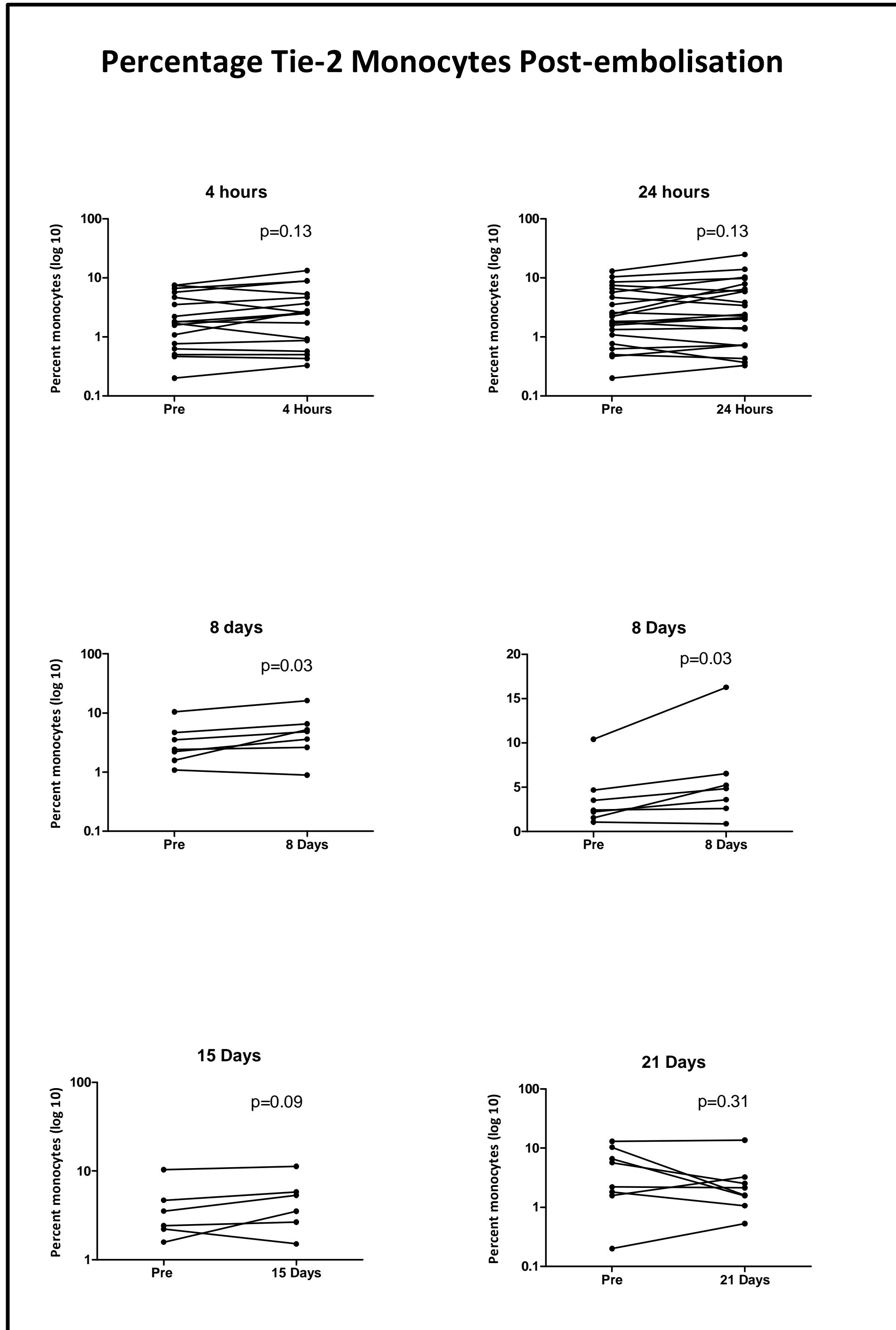
EDTA-preserved peripheral blood was collected for Tie-2 monocytes pre-embolisation and at 4 and 24 hours, 8, 15 and 21 days. Not all patients provided blood samples so the baseline values are given for each time-point with the patient number. With Tie-2 post embolisation the only significant increase was seen at day 8 post procedure in this small study group. This has been represented by both a Table (Table 5.3) and a Figure (Figure 5.5). Although the p-value becomes significant at day 8 the significance of this is unclear.

Table 5.3 Percentage of Tie-2 monocytes post embolisation
Values for Tie-2 monocytes are given in percentages of the monocyte population. Each post-embolisation value was compared individually to its pre-embolisation value to act as its own control using the Mann-Whitney U Test. The total value given is the median.

TIME	**PRE**	**4 HRS**	**PRE**	**24 HRS**	**PRE**	**8 DAYS**	**PRE**	**15 DAYS**	**PRE**	**21 DAYS**
No HCC patients	**17**		**22**		**8**		**6**		**8**	
Tie-2 monocytes	1.78	*2.55* *p=0.13*	2.02	2.33 *p=0.13*	**2.42**	**4.86** ***p=0.03***	2.98	4.44 *p=0.09*	3.96	1.86 *p=0.31*

Figure 5.5 Percentage of Tie-2 Monocytes Post-embolisation

Graphs use a Log scale due to wide range of data values. Day 8 presented as both linear and log scale.



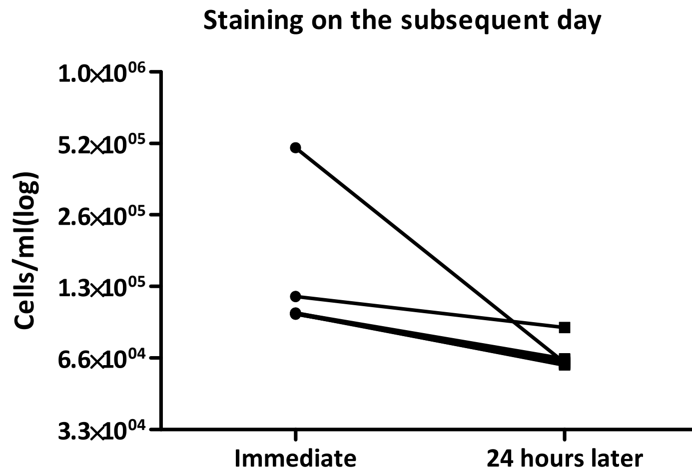
A control group of 5 patients undergoing cardiac angiography showed no increase in Tie-2 at 4 hours post procedure (0.4 versus 0.33 Percent monocytes p=0.58) but it was not possible to test later time points.

5.5.6 Endothelial Progenitor Cells

5.5.6.1 Assessing sample storage

The samples were either stained and analysed immediately or left overnight to be stained and analysed in the morning. Again the readings were unreliable if left overnight. The graph below uses a log scale due to the range of values.

Figure 5.6 Assessing re-staining on the subsequent day
Total number of cellular events counted by the flow cytometer when analysed immediately and at 24 hours after collection.

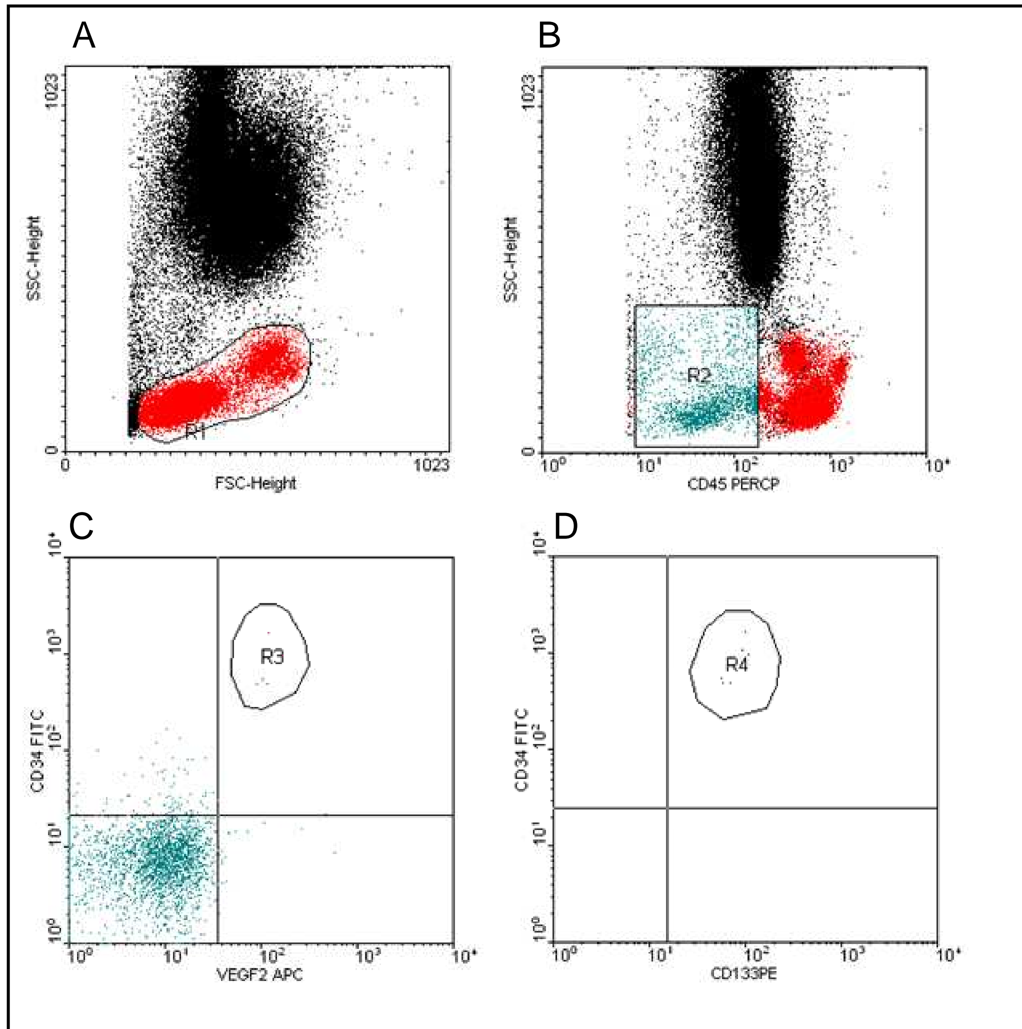


5.5.6.2 Endothelial Progenitor cells at baseline

Method One:

Figure 5.7 demonstrates the presence of cells defined as endothelial progenitor cells by the expression of CD34, CD133 and VEGFR2. According to this method EPCs formed 0.015% of mono-lymphocytic cells (range 0.0018-0.05) and 0.79% of CD45 low mono-lymphocytes (range 0.47-1.25) in a cohort of seven patients attending the oncology clinic. Four patients with HCC had samples analysed with similar values (0.013% of mono-lymphocytic cells; range 0.006-0.022%).

Figure 5.7 Initial gating strategy for the identification of EPCs



A: Gating around monocytes and lymphocytes using physical characteristics

B: CD45 negative / low cells identified.

C: Cells that are CD34 and VEGFR2 positive gated for

D: Confirming that they are also CD133 positive.

Three patients had samples taken at 24 hours post-embolisation with all three having a rise in the percentage of EPC's from a mean of 0.64% of CD45 positive events to 0.99% of CD45 positive events. The extremely small sample size and the very low frequency of "positive" events makes this difficult to interpret.

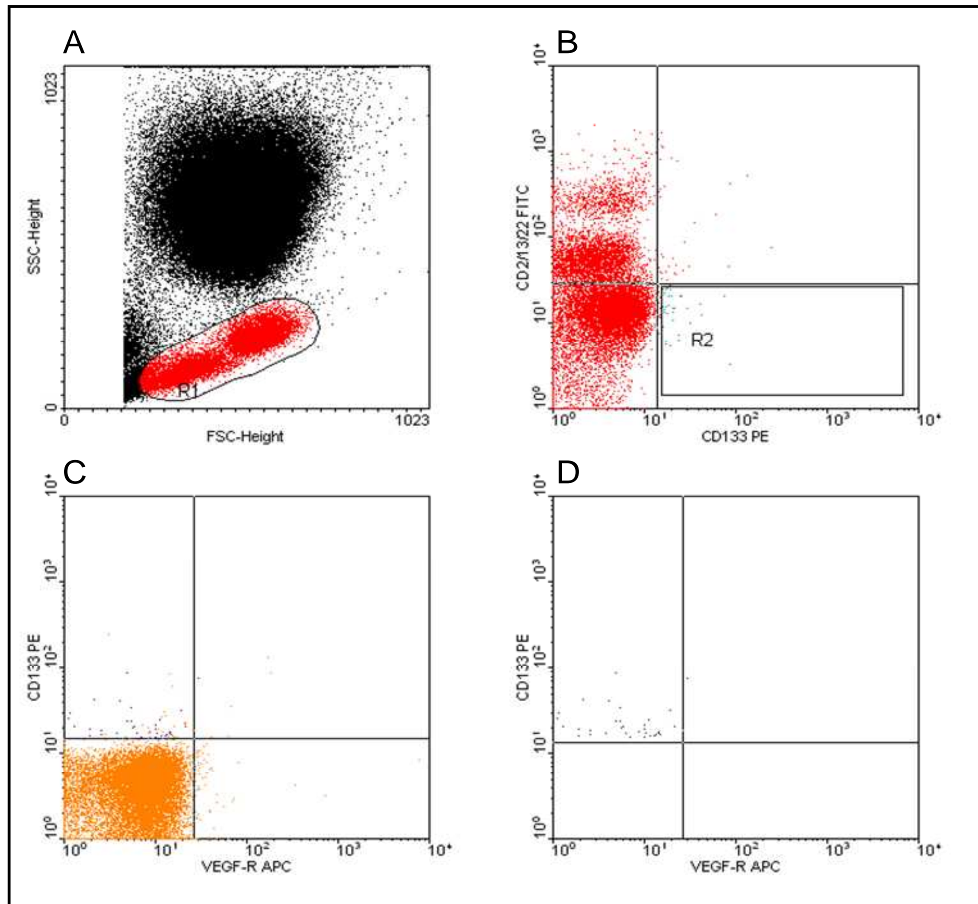
Method Two:

An alternative method based on a paper by Dulic-Sills et al (411) was then considered. This method used a “dumping” method to eliminate cells carrying the haematopoietic progenitor markers of CD2 / CD13 and CD22 before gating on the markers for endothelial progenitor cells, CD133 and VEGFR2. This “dumping” technique is the main difference from method one.

Initially 8 patients had blood tested using anonymous samples from an oncology clinic. These demonstrated that the presumed EPCs were present at 0.0023% of mono-lymphocytic cells (range 0-0.04).

In addition 3 patients with HCC had samples measured with this method giving a mean value of 0.0037% of mono-lymphocytic cells. After embolisation in 2 patients no rise was seen at 48 hours with a mean value of 0.005%. These values appear lower than previously published values; extrapolating to cells/mL would give values of 10-20 cells per ml. In addition cells were often only weakly positive for CD133 and VEGFR2.

Figure 5.8 Second gating strategy for the identification of EPCs



A = Isolating mononuclear and lymphocytic cells

B = Excluding cells that are positive for CD2 / CD13 and CD22 as well as selecting cells that are CD133 positive.

C = Selecting cells that are CD133 positive and VEGFR2 positive.

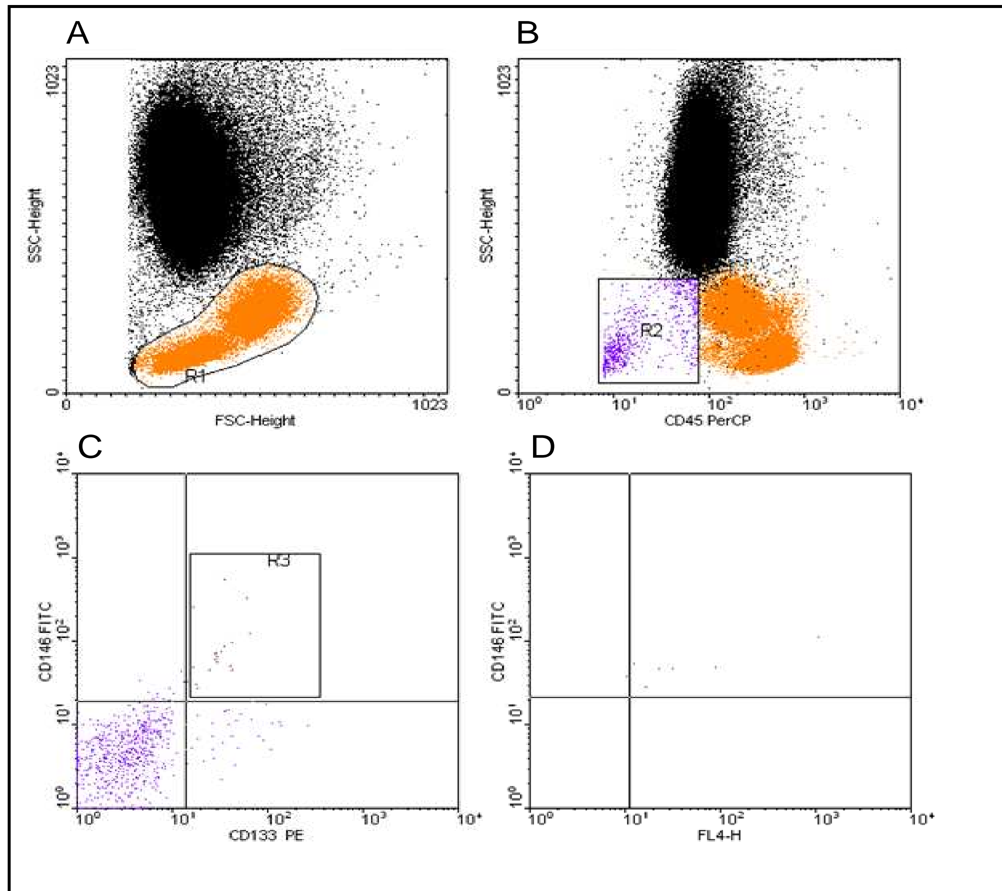
D = The final quadrant of CD133 and VEGFR2 positive cells contains 1 event.

Method Three

This method is similar to the first method but replaced CD34 with CD146. Although Asahara et al (171) used the combination of CD34 and VEGFR2 to characterise EPCs in 1997, the advent of CD133 to identify primitive stem cells meant CD34 was less useful. Cells that are CD133 positive and VEGR2 positive have been demonstrated to differentiate into endothelial cells (412), with functional properties of endothelial cells. CD34 expression on angiogenic cells is now considered to be variable. CD146 is an endothelial cell marker and a marker of mesenchymal stem cells and is thought to be expressed in EPCs.

Ten patients with HCC had blood analysed using this method resulting in a median value of 0.0046% of mono-lymphocytic cells (range 0.00097-0.1339) and 0.368% of CD45 low cells (range 0.094-3.82). This gives a median of 100 cells/ml (range 10-810). Using the adapted method on a further 6 patients with HCC and 3 healthy controls patients demonstrated a median value of 0.011% mono-lymphocytes in the patients with HCC compared to 0.0046% in the controls (p=0.024). This sample size is too small to make conclusions from but is consistent with other data from Poon et al (213).

Figure 5.9 Third gating strategy for the identification of EPCs



A = Isolating mono-lymphocytic cells

B = Identifying cells that are CD45 negative or weak positive

C = Isolating cells that are CD133 and CD146 positive

D = Isolating those that are CD146 and VEGF2 positive

5.5.6.3 EPC events over time post embolisation

Changes post-embolisation are detailed in Table 5.4. Ten patients had blood taken pre- and post-embolisation at 4 and 24 hours. In addition 3 patients had blood taken at 8 and 15 days post-embolisation. There was no evidence of a rise in EPC cells post-embolisation demonstrated in this small series. With the adoption of the third method the same pattern was seen with no statistical difference observed over time. This may be due to the small sample size. Of the 2 patients who had chemo-embolisation both had a rise in EPCs of 68% and 330% respectively at 4 hours before falling to just below baseline at 24 hours.

Table 5.4 EPC levels post embolisation

HCC PATIENTS	PRE	4	PRE	24 HRS	PRE	8 DAYS	PRE	15 DAYS
	10		**10**		**3**		**3**	
Median EPCs (% of ML cells)	0.0047	0.009 *p=0.69*	0.0047	0.0058 *p=0.43*	0.0049	0.0053 *p=0.75*	0.0049	0.0069 *P=0.5*

5.6 Conclusions

The aim of this chapter was to consider whether Tie-2 positive monocytes or endothelial progenitor cells could be investigated as biomarkers for the vascular disrupting agents. Our study was designed as a pilot study only, to assess the feasibility of these approaches rather than to provide definitive evidence for their suitability as biomarkers. As a pilot study, only a small number of patients were enrolled over a 2 year period making the data difficult to interpret and any conclusions only tentative. Future studies are aimed to investigate promising biomarkers on a larger scale. Ideally this project could recruit alongside future trials of TAE in malignancy as part of a multi-centre study. As flow cytometry cannot be done on stored blood any trial would of necessity be prospective

Tie-2 monocytes are known to localize to sites of angiogenesis (159) under the influence of angiopoietin-2 (158). The acute hypoxia from TAE was hypothesized to lead to cytokine release and secondary recruitment of Tie-2 positive monocytes.

Although there has been interest in targeting tumours using Tie-2 positive monocytes, there is little data on its use as a biomarker. In this study we considered it in patients with hepatocellular cancer undergoing embolisation. Our study was confounded by finding that values for Tie-2 positive cells varied between flow cytometers. Initial data recorded on the FACSCalibur machine gave a median value of 4.69% of monocytes compared to a value of 0.67% for the BD FACSAria. The difference may reflect reduced staining of a new batch of the Tie-2 antibody (personal communication R&D 2008). The analysis was therefore confined to data obtained from the FACSCalibur since the values corresponded to previous data published by Venneri et al.

There was no evidence in our data set that Tie-2 positive monocytes were statistically more frequent in patients with HCC as compared to healthy controls. There was a statistically significant correlation with AFP but not with liver function according to Child-Pugh status. Tie-2 monocytes did correlate with VEGF but there was no clear correlation between serum angiopoietin-2 and circulating Tie-2 monocytes ($r = 0.013^2$). Post-embolisation a rise in Tie-2 monocytes was seen with statistical significance reached by day 8. This could reflect recruitment from the bone marrow into the circulation. Recruitment may be hypoxia driven which could explain the correlation seen with serum VEGF. No relationship was seen with prognosis.

This was a pilot study to consider the feasibility of investigating Tie-2 monocytes further as a biomarker. One challenge is the possible variability between operators. As expression of Tie-2 is a continuous variable there may be no clear cut-off between positive and negative events (defining a cell as Tie-2 positive or negative). The other cell populations negative for Tie-2 can assist in defining a boundary and computer programs may aid visual inspection but subjectivity may remain. This work could be extended to include larger number of patients since the sample size was very small. It would also be possible to assess the soluble Tie-2 receptor in serum which has recently been shown to be elevated in neuroendocrine tumours and to correlate with serum angiopoietin-2 (166).

A second pilot experiment explored the use of EPCs as a possible biomarker. EPCs are known to be recruited from the bone marrow by angiogenic cytokines such as VEGF, bFGF, SDF-1 and GM-CSF (203). They migrate to the hypoxic tumour peripheries in areas of intense angiogenesis (413) as demonstrated by MRI tracking of labelled CD133 positive cells. The ablation of EPCs reduces tumour growth and vascularity (205). Previous work has shown they are elevated in hepatocellular cancer (194). Poon et al has shown that an elevated level of circulating EPCs correlated with tumour aggressiveness using a method

of scoring colony forming units (CFU's) in the peripheral blood and quantified with FACS (using VEGFR2, CD34 and CD133). EPC levels correlated with serum AFP, plasma VEGF and interleukin-8 and were higher in patients with non-resectable disease and in patients who recurred after potentially curative surgery (213). This work suggests EPCs as a potential biomarker in HCC. In breast cancer they have been used as a surrogate biomarker for an anti-angiogenesis drug (414). Our hypothesis was that the acute hypoxia resulting from tumour embolisation would lead to EPC mobilisation.

Shaked et al (202) demonstrated rapid mobilisation of EPCs after VDA treatment in a mouse model. The cells homed to the viable rim of tumour tissue. This increase could be blocked by a VEGFR neutralising antibody with a resulting increase in anti-tumour activity. After VDA administration an increase in VEGF, G-CSF and SDF-1 was seen. G-CSF contributed to EPC mobilisation and was induced by it. In human subjects plasma G-CSF, VEGF and SDF-1 increased 4 hours after administration of a VDA as measured by ELISA and fell again by 24 hours (203).

One of the difficulties with research in this area is the lack of a standard methodology to define an EPC by flow cytometry. In this study I considered three different methods. Initially I used a method based on the original data from Asahara et al (171) who used co-expression of CD34 and VEGFR2 to define an EPC. Later work questioned the reliance on the haematopoietic stem cell marker, CD34 (410) since it is found on both EPCs and more mature endothelial cells. I therefore explored the method described by Macey et al (411) which excluded cells on the basis of their expression of haematopoietic markers CD2 / CD13 and CD22 before selecting cells with the stem cell marker CD133 and VEGFR2. Using this method I found very low numbers of cells with weak staining for VEGFR2 and CD133.

The final method used the markers CD133 and CD146 instead of CD34, together with VEGFR2. Using this method we considered patients pre- and post-embolisation to assess for changes over 4 and 24 hours. In our small patient sample there was no indication of any difference. This differs from data published by Shaked et al which demonstrated a rise in EPCs at 4 hours which resolved by 24 hours (203). Their method differed from ours using $CD13^{+}VEGFR2^{+}CD45^{-/dim}CD117^{+}$. CD13 is known to be expressed on haematopoietic progenitor cells and was one of the markers used to exclude cells by Macey et al (411). As both VEGR2 and CD117 are also expressed (178) on subsets of haematopoietic progenitor cells it is likely they are included in the analysis. We used the same time points post embolisation as Shaked et al did post VDA. It may be that the EPC response to hypoxia induced by embolisation differs from that post VDA with a different time-course. Alternatively the rise post VDA may not be induced by tumour hypoxia but be secondary to an independent systemic effect of the VDA on bone marrow inducing EPC recruitment.

EPCs have potential as biomakers for anti-angiogenic agents. Challenges remain with the lack of a standard methodology to identify them. Flow cytometry is a valuable tool to identify candidate cells but unless they are cultured it is difficult to be certain they are indeed EPCs. The combination of $CD133^{+}$ and $VEGFR2^{+}$ has been validated by isolating and culturing these cells and demonstrating differentiation into endothelial cells with the functional properties of EPCs (412). In other papers CD34 has been reported as both positive and negative in EPCs (410, 415) as has CD45 (171, 415, 416), which may relate to lack of clarity of what constitutes dim expression. To avoid using CD45 Khan et al (410) recommends the use of an exclusionary gating strategy such as CD3 / CD19 and CD33. An alternative would be the gating strategy we employed in our second method of CD2 / CD13 and CD22.

The different methodologies employed suggest there are likely to be separate groups of cells with endothelial progenitor like properties and overlapping phenotypes. Some cells may be derived from monocytes or have monocytoid properties (417). Gulati et al (418) considered several cell types having the potential to differentiate into EPCs including monocytes expressing CD14 and those expressing CD133 and VEGFR. A clearer understanding of the differing cell types with angiogenic potential would be useful in determining their best role as biomarkers. To this aim Mellick et al (419) used the transcription factor inhibitor of DNA binding 1 in mouse transgenic models and tracked EPCs from bone marrow into circulation and then to tumour stroma. Ablation with a suicide gene led to a reduced tumour growth secondary to an impaired angiogenesis.

Further practical considerations with circulating EPCs are their rarity making it difficult to exclude the impact of background noise. In our study we aimed to collect 10^6 events in total. It is likely that collecting even larger number of events would be optimal with such rare-event analysis. The lengthy sorting time required may restrict EPC use to a research setting. Further work is needed to explore the relationship between EPC and malignancy.

Chapter Six: Conclusions

In this, my final chapter, I shall aim to summarise the key findings of my thesis and then consider them further in the context of the expanding field of radioimmunotherapy.

The initial hypothesis was that single agent radioimmunotherapy would be effective and relatively non-toxic in haematological malignancy. This statement appears consistent with the findings of Chapter Two, our Phase I study of ^{131}I-CHT25 in CD25-expressing lymphoma. This study has been published and is now progressing to a Phase Two to further assess efficacy and toxicity in a larger patient group.

Our initial hypothesis considered that solid tumours were more likely to present challenges for single-agent RIT. To date results have remained disappointing, particularly in advanced disease (94). To exploit synergy seen in animal models (123), RIT was combined with a VDA to induce tumour vascular shut-down. However as reported in Chapter Three, results remained disappointing with only minor evidence of efficacy and unexpected toxicity. The lack of efficacy may at least be partly attributed to the lack of vascular shut-down induced by CA4P on DCE-MRI.

This observation emphasised the need for an effective biomarker for the extent of vascular shut-down achieved by the VDA's. There are a number of limitations with imaging methods. Serum biomarkers have the advantage of being quantifiable, less operator-dependent, and repeatable. A biomarker would allow an optimal protocol to be developed to allow maximum synergy to occur and to minimise the risk of vascular shut-down preventing RIT access to tumour.

Chapters 4 and 5 report pilot studies on various compounds as potential biomarkers.

Two hypotheses were considered in Chapters 4 and 5. Both are investigated in preliminary pilot studies to consider whether further more definitive work would be feasible and desirable. The first hypothesis was that an appropriate model for vascular shut-down secondary to a VDA would be vascular shut-down secondary to physical means (TAE). Both are known to cause ischaemic necrosis with secondary hypoxia. TAE has the advantage of larger patient numbers and a more consistent time and level of vascular occlusion.

The second hypothesis was that this hypoxia would generate the secretion of angiogenic cytokines, Tie-2 expressing monocytes and Endothelial Progenitor Cells (EPC's) and that these would have potential to act as biomarkers. As a pilot study only, definitive answers were not anticipated but promising substances could be considered for further investigation in multi-centre studies. Evaluating our results in the context of prior research is important for our small studies. I shall then consider how this research relates to future work.

Currently RIT has been most successful in haematological malignancy, as predicted by the radiobiology. Much of the previous research has been in indolent B-cell lymphomas and targeted the CD20 cell surface antigen. This research has led to 2 licensed agents, ^{90}Y-ibritumomab tiuxetan (Zevalin®) (74) and ^{131}I-tositumomab (Bexxar®) (84). Both have shown clinical benefit at non-myeloablative doses. Although most patients relapse within 6-15 months (420) approximately 15-20% attain long term remissions and a proportion may be cured. RIT with more aggressive lymphomas, such as DLBCL, have typically been less successful, attributed to their higher proliferative rate failing to be controlled by the low dose rate of RIT.

Our study of ^{131}I-CHT25 in CD25-expressing lymphoma (mainly Hodgkin's lymphoma) demonstrated that, at the MTD, it was relatively well tolerated and showed some evidence of efficacy. The single-agent efficacy observed here is encouraging considering the higher proliferative rate of HL or T-cell lymphoma compared with indolent lymphoma. The toxicity profile was similar although a high rate of non-neutropenic infection was seen, whether this was due to an immunomodulatory effect of the RIC or due to the heavily pre-treated patient group is not clear.

Previous attempts at RIT in HL have used murine antibodies against the CD30 receptor (237) with limited evidence of efficacy. Our paper is important as it uses a chimeric antibody and demonstrates manageable toxicity. Although chimeric and humanized antibodies (233-234) have been used in their unlabeled form in HL, concerns about their longer half-lives exposing patients to greater bone marrow suppression have so far limited their use in RIT. Our study also targets CD25, a receptor which has not been explored as a target in HL, although Waldmann et al have investigated it in ATLL (244). The advantage of targeting CD25 over CD30 may relate to its expression on the surrounding inflammatory cells as well as malignant cells. These inflammatory cells are thought to consist of predominantly CD25 positive T-regulatory cells that may protect the malignant cells from the host response and aid their malignant potential (255). Further studies will aim to clarify the immunomodulatory effect of ^{131}I-CHT25, particularly on these T-regulatory and Natural Killer cells.

Due to the results of the Phase I study presented here, ^{131}I-CHT25 is due to enter further Phase II trials in patients with relapsed HL. This trial should help determine whether single-agent RIT can deliver long-term remissions in HL. However if the experience of indolent B-cell lymphomas are typical most patients will either fail to respond or have short-lived responses therefore improving RIT remains vital. Different strategies can be considered. RIT may be

more effective if combined with chemotherapy or other targeted agents, if given with high-dose therapies such as ASCT or allogeneic transplant, if it is given earlier in the disease course, if multiple antigens are targeted or if pre-targeting two-step methods are used to increase the dose delivered to tumour. Methods to better predict haematological toxicity such as FLT-3L may aid dosing and even allow dose escalation in a sub-group of patients (254).

Our Phase II study will plan to investigate some of these strategies. It will administer unlabeled CD25 antibody prior to the RIC to remove any 'sink' of soluble IL-2 from the circulation. The initial Phase II study will remain in patients with relapsed HL but eventually it may be feasible to administer it earlier, either in first relapse in high-risk patients with HL or first-line in patients with poor prognosis T-cell lymphoma. Using this strategy in NHL Kaminski et al improved response rate, but more importantly increased the number of durable responses, with a time to progression of over 5 years (83).

RIT can be combined with chemotherapy or other targeted agents. Press et al administered RIT after standard chemotherapy for follicular lymphoma and found an increase in CR and molecular remission rate with little additional toxicity (421) producing a 5 year progression free survival of 67%. Combinations include either ASCT or allogeneic bone marrow transplants. With allogeneic transplants Press et al has replaced total body irradiation used in conditioning regimens with myeloablative RIT. Results have been promising with high and durable responses rates (422) including in patients with DLBCL. RIT can be included as part of the conditioning for autologous transplantation. Devizzi et al has published interesting results in a range of B-cell lymphomas (423) and a phase III study is currently underway considering the addition of ^{131}I-tositimomab to a standard autologous regimen in DLBCL. In our own study 2 patients went on to receive high-dose therapy (1 autograft; 1 allograft). No excess toxicity was seen and both transplants engrafted satisfactorily. We

intend to further investigate the potential of ^{131}I-CHT25 in the conditioning regimens of ASCT in relapsed HL.

In contrast to haematological malignancy, radiobiology predicted that single-agent RIT is likely to be disappointing in solid tumours, particularly in patients with bulky, resistant disease (94). A variety of methods have been attempted to improve RIT. The development of antibody fragments has improved tumour uptake and clearance (45), chimeric and humanized antibodies have reduced immunogenicity (54), pre-targeting two-step methods have allowed an improved tumour to blood ratio (60) and various combinations of agents have been tried including chemotherapeutics and cytokines. Our group hoped to exploit the synergy seen in animal models between RIT and the VDA's to improve the efficacy of RIT in solid tumours (119).

The ^{131}I-A5B7 Phase II study is reported in Chapter 3. Synergy between the RIT and VDA was anticipated due to their differing mechanisms and sites of action, with the VDA targeting the poorly perfused centre and the RIT the tumour rim. In animal models a sub-curative therapy was transformed into a curative one with this combination. No unacceptable toxicities were seen (119). Although no additive toxicity was anticipated in this study the starting doses were below the MTD's derived from the single agent data. However dose-limiting myelosuppression was seen with the first cohort. Even after reducing the administered activity further toxicity occurred and the study was stopped.

This toxicity was unexpected. It may reflect an independent cytotoxic effect of the CA4P similar to other chemotherapeutics that interact with tubulin such as the vinca alkaloids (277). The combination of a VDA with RIT is novel so there are no equivalent clinical trials to compare to. However a significant myelosuppressive effect has not been identified in any of the single agent studies (265-267) or when in combination with chemotherapy (279) apart from

a single study where CA4P was administered 60 minutes post carboplatin. The subsequent vascular shut down was thought to impede the renal clearance of the carboplatin (280). There was no evidence from our study that the pharmacokinetics of the ^{131}I-A5B7 were altered by the CA4P when comparisons were made with the single agent study (94) or that the administration of the CA4P two weeks prior to the first infusion of the RIC had any impact on tumour uptake. It is possible that the pharmacokinetics of the CA4P administered post RIC administration were affected since data was collected from the preceding administration due to radiation protection issues. It may simply represent the more heavily pre-treated patient group when compared to earlier studies when fewer chemotherapeutic agents were available (94).

No objective responses were seen although one patient who had a reduction in blood flow on DCE-MRI post CA4P and a high tumour absorbed radiation dose did show a minor radiological response associated with a fall in tumour markers. The lack of responses may be secondary to the low VDA dose administered and a failure of adequate vascular shut-down. It may also be due to chance in a small patient group, more heavily pre-treated patients losing antigen expression secondary to tumour de-differentiation or the lower tumour absorbed dose compared to Lane et al (94) which may itself be secondary to de-differentiation. The problems with ensuring that the biological outcome of vascular shut down was met highlighted the necessity of an improved biomarker in these multi-modality trials.

The future of RIT combined with a VDA remains uncertain due to the unexpected toxicity encountered. Future animal work may help to delineate why this unexpected toxicity occurred. Currently multiple studies are in process investigating the combinations of a VDA with chemotherapy, anti-angiogenic agents and targeted agents. In addition newer, more potent VDA's are in development (424) that may induce vascular shut-down more efficiently. The

RIT may be delivered using pre-targeting methods where the delivery of the antibody is separated from that of the radioisotope. This has the advantage of improving the tumour to normal body ratio and reducing the risk of toxicity or of allowing a safer escalation of injected activity (60). Antibody engineering and the use of fragments including minibodies and SIP's may also help to increase tumour uptake and clearance and therefore improve the tumour to normal tissue ratios (50).

As described earlier one of the current difficulties of combining RIT with a VDA is the lack of a good biomarker to measure the effects on the vasculature. This is particularly problematic when issues of scheduling therapies are crucial to both efficacy and toxicity. Current imaging methods are time-consuming, costly and exclude many patients. They cannot be used in a radio-active patient after radioisotope administration. A reliable surrogate marker would be of tremendous benefit in drug development, particularly if a serum marker could be identified which was easily reproducible and quantifiable.

Chapters 4 and 5 present pilot studies considering the angiogenic cytokines, Tie-2 expressing monocytes and Endothelial Progenitor Cells as possible biomarkers for the VDA's and other agents causing vascular shut-down. Trans-arterial embolisation was used as a model for vascular shut down induced by the VDA's. It was hypothesised that TAE would induce ischaemic necrosis (141) leading to the release of HIF1-α and subsequently the angiogenic cytokines, EPC's and Tie-2 monocytes. TAE was chosen as a model due to its more consistent extent of and time duration of vascular shut-down together with the possibility of a larger patient group. Even as a pilot study it was anticipated it would take approximately 2 years to collect the data.

As a pilot study our data can not be considered definitive but instead aimed to provide directions for future research. Serum Angiopoietin-2 is known to be elevated in HCC (348) and be an independent prognostic factor. Our data confirms that of Scholz et al that Angiopoietin-2 levels did not correlate with tumour characteristics but did with markers of severity of liver disease (348). In addition our study found no difference in Ang-2 levels between patients with cirrhosis and those with HCC alone. This differs from Scholz et al and may represent chance in a small study. Alternatively it could reflect the differences between the patient groups, the respective AFP's suggest that Scholz et al includes more patients with an advanced tumour stage than in our study. The data presented here and in Scholz et al (348) suggest that the source of Angiopoietin-2 may not be tumour but the surrounding cirrhotic liver. Histological studies have been contradictory (346-347), which may relate to heterogeneity in tumour expression.

Serum angiopoietin-1 has been less studied than angiopoietin-2 and our study is the first I am aware of in HCC. I report a lower level of serum Ang-1 in patients with HCC compared to healthy controls. The high Ang-2 and low Ang-1 levels are consistent with a pro-angiogenic environment. Tissue expression studies have either found a stable level of Ang-1 ((331, 334) or an elevated one (306, 425) although in all these studies the Ang2:Ang1 ratio was elevated. It is not clear what causes the discrepancy between these findings; further studies on serum Angiopoietin-1 in HCC are indicated. Of note Park et al did find a reduced serum level in NSCLC (342). In our study serum Ang-1 was not of prognostic significance and no difference was observed in serum levels post-embolisation suggesting Ang-1 may be derived at least partly from the surrounding cirrhotic liver. This finding does fit in with the expression data where levels between tumour and cirrhotic liver were similar (331).

Studies have shown an elevated serum VEGF in patients with HCC (307). Higher levels were associated with a poorer prognosis post TAE (309). In a meta-analysis of 8 studies, all but 1 in the Asian population, serum VEGF was associated with a poorer outcome (300). However in our study no difference could be determined between serum VEGF in HCC and healthy controls. Although HCC values appeared consistent with those obtained in Poon et al (309) the control values were higher. This highlights the difficulty of using serum VEGF which is known to have a comparatively wide range in the normal healthy population, a challenge for small studies such as ours. Our study did find a poorer prognosis for patients with high serum VEGF levels consistent with the meta-analysis (300). Although serum VEGF rose post embolisation the rise in our study was relatively late, becoming significant by day 15. Other studies have shown an earlier rise by day 7 (395).

Few studies have considered bFGF in HCC and they all are in the Asian population. Our data confirms that from Poon et al (364) who did not find any difference between serum bFGF in patients with HCC and controls. However their group found a correlation between high bFGF levels and a poorer outcome which in common with another group (365) we could not replicate. Values did not change post-embolisation and no other studies have considered this.

Erythrocytosis is relatively common in patients with HCC (2-12%) (406) and thought to be secondary to an elevated erythropoietin level. Few studies have assessed serum erythropoietin levels in HCC and those that do tend to consist of case studies or small series (390, 406). Our data is consistent with that work, showing erythropoietin levels are elevated in HCC and in patients with cirrhosis alone. In our small data series levels did not seem to affect prognosis. Levels consistently doubled post-embolisation remaining elevated for 1 week. This rise is likely secondary to vascular shut down causing ischaemia and a smaller rise

was seen post coronary angiography. I am aware of no other study that has considered erythropoietin levels post TAE.

My hypothesis with the angiogenic cytokines was that the ischaemic necrosis induced by embolisation would cause them to be secreted secondary to hypoxia. Their rise would therefore be a biological marker for the extent of vascular shutdown. Serum angiogenic cytokines have the advantage of being determined by ELISA, which is a relatively straightforward validated assay and allows samples to be stored and analysed later.

Of the angiogenic cytokines studied serum erythropoietin has the most potential as a biomarker with a consistent rise post embolisation. The Angiopoietins failed to rise and may be secreted by the surrounding cirrhosis rather than the tumour as did bFGF. Although VEGF levels did increase they did so late and remained very variable between different patients making them less useful. This study is only a pilot, so potential biomarkers need to be further evaluated in larger series. To expand patient numbers this may require multi-centre studies and include trials of the VDA's as they move into later phase clinical studies. Whilst erythropoietin merits further evaluation other substances are interesting. These include TGF-beta1 and its co-receptor CD105 which are thought to enhance the malignant potential and angiogenesis (426). Shaked et al have considered SDF-1 (stromal derived factor-1) and G-CSF (granulocyte colony-stimulating factor) (203) post VDA in both animal models and patients and demonstrated rapid rises within 4 hours of administration.

Tie-2 monocytes are known to be recruited from bone marrow secondary to hypoxia and directed to sites of angiogenesis under the influence of angiopoietin-2 (158). This led to the hypothesis that they may have potential as a biomarker for vascular shut down. Although Tie-2 monocytes have been detected in the blood of patients with a variety of malignancies including lung,

kidney, colon, pancreas and neuroendocrine tumours (165) they have not been studied in hepatocellular cancer or been investigated as a biomarker. Unfortunately our study was limited by poor Tie-2 staining (R&D Personal Communication) limiting patient numbers to 16. In this very small cohort, levels of Tie-2 monocytes were not elevated in patients with HCC, although they were higher in HCC patients with an elevated AFP. A correlation was seen between serum VEGF and Tie-2 and both rose from day 8 post-embolisation. This late rise may reflect their recruitment induced by hypoxia. Both VEGF and Tie-2 monocytes are more likely to be responding to the same stimulus rather than being dependent on each other since in-vitro work has not shown Tie-2 recruitment to be dependent on VEGF (427). Although these observations are interesting for further study they cannot be considered definitive due to the extremely small sample size and the lack of other corroborating studies in this area.

Our study demonstrates the feasibility of using flow cytometry to assess Tie-2 monocytes and found these cells present in HCC. Future larger studies would be required to optimize and standardize the flow cytometry protocol and then investigate their role as a potential biomarker for vascular shut down more definitively. Tie-2 expressing monocytes have the ability to hone to tumours. Studies are currently in progress using them as delivery agents for cytotoxics (167).

Considerable controversy exists about endothelial progenitor cells and the role they play in promoting tumour angiogenesis. They are thought to be recruited to tumours under the influence of the angiogenic cytokines and secondary to hypoxia. A single study by Ho et al (213) has shown they are elevated in HCC and correlate with a poorer prognosis. In mouse models levels have been shown to increase at 4 hours post VDA administration together with VEGF (203).

Our study could not replicate these findings. This may be due to the small sample size of our study together with the variability in EPC levels between patients. A rise in EPC levels may be a transient event and its time-course may differ after administration of a VDA or post-TAE. It may also be explained if the rise post VDA was not secondary to hypoxia but a direct effect of the drug on the bone marrow.

One of the difficulties currently with EPC assessment is the lack of a standard methodology to define them. There is currently no universally accepted combination of cell surface markers that define an EPC and different groups use different combinations that are often mutually exclusive. For example Shaked et al (203) reported that EPCs rose post VDA administration. But the combination of cell markers selected would also select subgroups of haematopoietic progenitor cells and would have been excluded by Macey et al (411). Therefore it is difficult to compare different papers as they are often not comparing the same cell population.

Before EPC's can be considered as a viable proposition as a biomarker more information is therefore needed to define what is an endothelial progenitor cell. Currently combinations used should be restricted to those that have been validated with cell culture work such as the combination of CD133 and VEGFR2 (171), and found to have the functional properties of EPC's. Future work considering the genetic differences between these overlapping cell populations look may provide some clarity (419).

In practical terms EPC acquisition involves rare event analysis with a resulting difficulty in excluding background noise from the data. Large numbers of events are required which take a long time to acquire. The lack of true clustering of events means automation is difficult and variability between operators can

introduce bias. These difficulties reduce its potential as a biomarker for standard clinical practice.

In summary and with consideration of both my own studies presented here and the previous published research RIT remains a promising treatment for haematological malignancy. The ^{131}I-CHT25 clinical trial demonstrated efficacy and was relatively well tolerated at the MTD. This trial is now entering recruitment as part of Phase II. In contrast RIT in advanced solid tumours has remained disappointing. My thesis describes a novel approach to this problem, of combining a VDA with RIT to exploit the synergy previously noted in animal models. Unfortunately unexpected toxicity was seen whilst the dose of CA4P remained sub-optimal to allow effective vascular shutdown. The limited shutdown may have contributed to the lack of clinical efficacy. The problems of combining these two agents have highlighted the need for an effective biomarker for the VDAs. I have considered the angiogenic cytokines, Tie-2 expressing monocytes and the endothelial progenitor cells in preliminary feasibility studies using TAE as a model. The study identified promising compounds to investigate further in future studies.

Reference List

(1) Mathis KL, Nelson H, Pemberton JH, Haddock MG, Gunderson LL. Unresectable colorectal cancer can be cured with multimodality therapy. Ann Surg 2008 Oct;248(4):592-598.

(2) Tannock IF. Conventional cancer therapy: promise broken or promise delayed? Lancet 1998 May;351 Suppl 2:SII9-16.

(3) Samson MK, Rivkin SE, Jones SE, Costanzi JJ, LoBuglio AF, Stephens RL, et al. Dose-response and dose-survival advantage for high versus low-dose cisplatin combined with vinblastine and bleomycin in disseminated testicular cancer. A Southwest Oncology Group study. Cancer 1984 Mar 1;53(5):1029-1035.

(4) Rivera GK, Raimondi SC, Hancock ML, Behm FG, Pui CH, Abromowitch M, et al. Improved outcome in childhood acute lymphoblastic leukaemia with reinforced early treatment and rotational combination chemotherapy. Lancet 1991 Jan 12;337(8733):61-66.

(5) Hanrahan EO, Broglio K, Frye D, Buzdar AU, Theriault RL, Valero V, et al. Randomized trial of high-dose chemotherapy and autologous hematopoietic stem cell support for high-risk primary breast carcinoma: follow-up at 12 years. Cancer 2006 Jun 1;106(11):2327-2336.

(6) Mort D, Lansdown M, Smith N, Protopapa K, Mason M. Systemic Anti-Cancer Therapy: For better, for worse? 2008 Nov. Report No.: 1.

(7) Wilder RB, Denardo GL, DeNardo SJ. Radioimmunotherapy: recent results and future directions. J Clin Oncol 1996 Apr;14(4):1383-1400.

(8) Belyakov OV, Mitchell SA, Parikh D, Randers-Pehrson G, Marino SA, Amundson SA, et al. Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. Proc Natl Acad Sci U S A 2005 Oct 4;102(40):14203-14208.

(9) Lim SM, Denardo GL, DeNardo DA, Shen S, Yuan A, O'Donnell RT, et al. Prediction of myelotoxicity using radiation doses to marrow from body, blood and marrow sources. J Nucl Med 1997 Sep;38(9):1374-1378.

(10) Srivastava S, Dadachova E. Recent advances in radionuclide therapy. Semin Nucl Med 2001 Oct;31(4):330-341.

(11) Humm JL. Dosimetric aspects of radiolabeled antibodies for tumor therapy. J Nucl Med 1986 Sep;27(9):1490-1497.

(12) Koppe MJ, Bleichrodt RP, Soede AC, Verhofstad AA, Goldenberg DM, Oyen WJ, et al. Biodistribution and therapeutic efficacy of (125/131)I-, (186)Re-, (88/90)Y-, or (177)Lu-labeled monoclonal antibody MN-14 to carcinoembryonic antigen in mice with small peritoneal metastases of colorectal origin. J Nucl Med 2004 Jul;45(7):1224-1232.

(13) O'Donoghue JA, Bardies M, Wheldon TE. Relationships between tumor size and curability for uniformly targeted therapy with beta-emitting radionuclides. J Nucl Med 1995 Oct;36(10):1902-1909.

(14) Denardo GL, DeNardo SJ, O'Donnell RT, Kroger LA, Kukis DL, Meares CF, et al. Are radiometal-labeled antibodies better than iodine-131-labeled antibodies: comparative pharmacokinetics and dosimetry of copper-67-, iodine-131-, and yttrium-90-labeled Lym-1 antibody in patients with non-Hodgkin's lymphoma. Clin Lymphoma 2000 Sep;1(2):118-126.

(15) Boswell CA, Brechbiel MW. Development of radioimmunotherapeutic and diagnostic antibodies: an inside-out view. Nucl Med Biol 2007 Oct;34(7):757-778.

(16) Carrasquillo JA, White JD, Paik CH, Raubitschek A, Le N, Rotman M, et al. Similarities and differences in 111In- and 90Y-labeled 1B4M-DTPA antiTac monoclonal antibody distribution. J Nucl Med 1999 Feb;40(2):268-276.

(17) Jurcic JG, Larson SM, Sgouros G, McDevitt MR, Finn RD, Divgi CR, et al. Targeted alpha particle immunotherapy for myeloid leukemia. Blood 2002 Aug 15;100(4):1233-1239.

(18) Allen BJ, Raja C, Rizvi S, Song EY, Graham P. Tumour anti-vascular alpha therapy: a mechanism for the regression of solid tumours in metastatic cancer. Phys Med Biol 2007 Jul 7;52(13):L15-L19.

(19) McDevitt MR, Sgouros G, Finn RD, Humm JL, Jurcic JG, Larson SM, et al. Radioimmunotherapy with alpha-emitting nuclides. Eur J Nucl Med 1998 Sep;25(9):1341-1351.

(20) Adams GP, Shaller CC, Chappell LL, Wu C, Horak EM, Simmons HH, et al. Delivery of the alpha-emitting radioisotope bismuth-213 to solid tumors via single-chain Fv and diabody molecules. Nucl Med Biol 2000 May;27(4):339-346.

(21) Raja C, Graham P, bbas Rizvi SM, Song E, Goldsmith H, Thompson J, et al. Interim analysis of toxicity and response in phase 1 trial of systemic targeted alpha therapy for metastatic melanoma. Cancer Biol Ther 2007 Jun;6(6):846-852.

(22) Kassis AI, Harapanhalli RS, Adelstein SJ. Strand breaks in plasmid DNA after positional changes of Auger electron-emitting iodine-125: direct compared to indirect effects. Radiat Res 1999 Nov;152(5):530-538.

(23) Anthony LB, Woltering EA, Espenan GD, Cronin MD, Maloney TJ, McCarthy KE. Indium-111-pentetreotide prolongs survival in gastroenteropancreatic malignancies. Semin Nucl Med 2002 Apr;32(2):123-132.

(24) Costantini DL, Chan C, Cai Z, Vallis KA, Reilly RM. (111)In-labeled trastuzumab (Herceptin) modified with nuclear localization sequences (NLS): an Auger electron-emitting radiotherapeutic agent for HER2/neu-amplified breast cancer. J Nucl Med 2007 Aug;48(8):1357-1368.

(25) Costantini DL, McLarty K, Lee H, Done SJ, Vallis KA, Reilly RM. Antitumor effects and normal-tissue toxicity of 111In-nuclear localization sequence-trastuzumab in athymic mice bearing HER-positive human breast cancer xenografts. J Nucl Med 2010 Jul;51(7):1084-1091.

(26) Llewelyn MB, Hawkins RE, Russell SJ. Discovery of antibodies. BMJ 1992 Nov 21;305(6864):1269-1272.

(27) Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975 Aug 7;256(5517):495-497.

(28) Jazirehi AR, Vega MI, Bonavida B. Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. Cancer Res 2007 Feb 1;67(3):1270-1281.

(29) Kim R, Emi M, Tanabe K. Cancer immunosuppression and autoimmune disease: beyond immunosuppressive networks for tumour immunity. Immunology 2006 Oct;119(2):254-264.

(30) Fowler JF. Radiobiological aspects of low dose rates in radioimmunotherapy. Int J Radiat Oncol Biol Phys 1990 May;18(5):1261-1269.

(31) Wheldon TE, O'Donoghue JA. The radiobiology of targeted radiotherapy. Int J Radiat Biol 1990 Jul;58(1):1-21.

(32) Griffith MH, Yorke ED, Wessels BW, Denardo GL, Neacy WP. Direct dose confirmation of quantitative autoradiography with micro-TLD measurements for radioimmunotherapy. J Nucl Med 1988 Nov;29(11):1795-1809.

(33) Bacher K, Thierens HM. Accurate dosimetry: an essential step towards good clinical practice in nuclear medicine. Nucl Med Commun 2005 Jul;26(7):581-586.

(34) Denardo GL, Siantar CL, DeNardo SJ. Radiation dosimetry for radionuclide therapy in a nonmyeloablative strategy. Cancer Biother Radiopharm 2002 Feb;17(1):107-118.

(35) Stabin MG, Sparks RB, Crowe E. OLINDA/EXM: the second-generation personal computer software for internal dose assessment in nuclear medicine. J Nucl Med 2005 Jun;46(6):1023-1027.

(36) Tagesson M, Ljungberg M, Strand SE. A Monte-Carlo program converting activity distributions to absorbed dose distributions in a radionuclide treatment planning system. Acta Oncol 1996;35(3):367-372.

(37) Kassis AI. The MIRD approach: remembering the limitations. J Nucl Med 1992 May;33(5):781-782.

(38) Rajendran JG, Fisher DR, Gopal AK, Durack LD, Press OW, Eary JF. High-dose (131)I-tositumomab (anti-CD20) radioimmunotherapy for non-Hodgkin's lymphoma: adjusting radiation absorbed dose to actual organ volumes. J Nucl Med 2004 Jun;45(6):1059-1064.

(39) Dewaraja YK, Wilderman SJ, Ljungberg M, Koral KF, Zasadny K, Kaminiski MS. Accurate dosimetry in 131I radionuclide therapy using

patient-specific, 3-dimensional methods for SPECT reconstruction and absorbed dose calculation. J Nucl Med 2005 May;46(5):840-849.

(40) Tuttle RM, Leboeuf R, Robbins RJ, Qualey R, Pentlow K, Larson SM, et al. Empiric radioactive iodine dosing regimens frequently exceed maximum tolerated activity levels in elderly patients with thyroid cancer. J Nucl Med 2006 Oct;47(10):1587-1591.

(41) Pauwels S, Barone R, Walrand S, Borson-Chazot F, Valkema R, Kvols LK, et al. Practical dosimetry of peptide receptor radionuclide therapy with (90)Y-labeled somatostatin analogs. J Nucl Med 2005 Jan;46 Suppl 1:92S-98S.

(42) Matthay KK, Panina C, Huberty J, Price D, Glidden DV, Tang HR, et al. Correlation of tumor and whole-body dosimetry with tumor response and toxicity in refractory neuroblastoma treated with (131)I-MIBG. J Nucl Med 2001 Nov;42(11):1713-1721.

(43) Dillman RO. Radiolabeled anti-CD20 monoclonal antibodies for the treatment of B-cell lymphoma. J Clin Oncol 2002 Aug 15;20(16):3545-3557.

(44) Dearling JL, Flynn AA, Qureshi U, Whiting S, Boxer GM, Green A, et al. Localization of radiolabeled anti-CEA antibody in subcutaneous and intrahepatic colorectal xenografts: influence of tumor size and location within host organ on antibody uptake. Nucl Med Biol 2009 Nov;36(8):883-894.

(45) Huhalov A, Chester KA. Engineered single chain antibody fragments for radioimmunotherapy. Q J Nucl Med Mol Imaging 2004 Dec;48(4):279-288.

(46) Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, et al. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. Proc Natl Acad Sci U S A 1988 Aug;85(16):5879-5883.

(47) Begent RH, Verhaar MJ, Chester KA, Casey JL, Green AJ, Napier MP, et al. Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. Nat Med 1996 Sep;2(9):979-984.

(48) Wu AM, Yazaki PJ. Designer genes: recombinant antibody fragments for biological imaging. Q J Nucl Med 2000 Sep;44(3):268-283.

(49) Yazaki PJ, Wu AM, Tsai SW, Williams LE, Ikler DN, Wong JY, et al. Tumor targeting of radiometal labeled anti-CEA recombinant T84.66 diabody and t84.66 minibody: comparison to radioiodinated fragments. Bioconjug Chem 2001 Mar;12(2):220-228.

(50) Sauer S, Erba PA, Petrini M, Menrad A, Giovannoni L, Grana C, et al. Expression of the oncofetal ED-B-containing fibronectin isoform in hematologic tumors enables ED-B-targeted 131I-L19SIP radioimmunotherapy in Hodgkin lymphoma patients. Blood 2009 Mar 5;113(10):2265-2274.

(51) Graff CP, Chester K, Begent R, Wittrup KD. Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. Protein Eng Des Sel 2004 Apr;17(4):293-304.

(52) Osbourn JK, Field A, Wilton J, Derbyshire E, Earnshaw JC, Jones PT, et al. Generation of a panel of related human scFv antibodies with high affinities for human CEA. Immunotechnology 1996 Sep;2(3):181-196.

(53) Adams GP, Schier R, McCall AM, Simmons HH, Horak EM, Alpaugh RK, et al. High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. Cancer Res 2001 Jun 15;61(12):4750-4755.

(54) Carter PJ. Potent antibody therapeutics by design. Nat Rev Immunol 2006 May;6(5):343-357.

(55) Ledermann JA, Begent RH, Massof C, Kelly AM, Adam T, Bagshawe KD. A phase-I study of repeated therapy with radiolabelled antibody to carcinoembryonic antigen using intermittent or continuous administration of cyclosporin A to suppress the immune response. Int J Cancer 1991 Mar 12;47(5):659-664.

(56) Sakahara H, Reynolds JC, Carrasquillo JA, Lora ME, Maloney PJ, Lotze MT, et al. In vitro complex formation and biodistribution of mouse antitumor monoclonal antibody in cancer patients. J Nucl Med 1989 Aug;30(8):1311-1317.

(57) Hasholzner U, Stieber P, Meier W, Lamerz R. Value of HAMA--determination in clinical practice--an overview. Anticancer Res 1997 Jul;17(4B):3055-3058.

(58) Hwang WY, Foote J. Immunogenicity of engineered antibodies. Methods 2005 May;36(1):3-10.

(59) Gautherot E, Bouhou J, Le Doussal JM, Manetti C, Martin M, Rouvier E, et al. Therapy for colon carcinoma xenografts with bispecific antibody-targeted, iodine-131-labeled bivalent hapten. Cancer 1997 Dec 15;80(12 Suppl):2618-2623.

(60) Goldenberg DM, Sharkey RM, Paganelli G, Barbet J, Chatal JF. Antibody pretargeting advances cancer radioimmunodetection and radioimmunotherapy. J Clin Oncol 2006 Feb 10;24(5):823-834.

(61) Sharkey RM, Goldenberg DM. Use of antibodies and immunoconjugates for the therapy of more accessible cancers. Adv Drug Deliv Rev 2008 Sep;60(12):1407-1420.

(62) Cairns R, Papandreou I, Denko N. Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment. Mol Cancer Res 2006 Feb;4(2):61-70.

(63) Fukumura D, Jain RK. Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. Microvasc Res 2007 Sep;74(2-3):72-84.

(64) Heldin CH, Rubin K, Pietras K, Ostman A. High interstitial fluid pressure - an obstacle in cancer therapy. Nat Rev Cancer 2004 Oct;4(10):806-813.

(65) Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001 Feb 21;93(4):266-276.

(66) Russell SJ, Llewelyn MB, Hawkins RE. Principles of antibody therapy. BMJ 1992 Dec 5;305(6866):1424-1429.

(67) Carter P, Smith L, Ryan M. Identification and validation of cell surface antigens for antibody targeting in oncology. Endocr Relat Cancer 2004 Dec;11(4):659-687.

(68) Maloney DG, Smith B, Rose A. Rituximab: mechanism of action and resistance. Semin Oncol 2002 Feb;29(1 Suppl 2):2-9.

(69) Maxwell P. Carcinoembryonic antigen: cell adhesion molecule and useful diagnostic marker. Br J Biomed Sci 1999;56(3):209-214.

(70) Cragg MS, Walshe CA, Ivanov AO, Glennie MJ. The biology of CD20 and its potential as a target for mAb therapy. Curr Dir Autoimmun 2005;8:140-174.

(71) Zhang Y, Pastan I. High shed antigen levels within tumors: an additional barrier to immunoconjugate therapy. Clin Cancer Res 2008 Dec 15;14(24):7981-7986.

(72) Press MF, Sauter G, Bernstein L, Villalobos IE, Mirlacher M, Zhou JY, et al. Diagnostic evaluation of HER-2 as a molecular target: an assessment of accuracy and reproducibility of laboratory testing in large, prospective, randomized clinical trials. Clin Cancer Res 2005 Sep 15;11(18):6598-6607.

(73) Behr TM, Salib AL, Liersch T, Behe M, Angerstein C, Blumenthal RD, et al. Radioimmunotherapy of small volume disease of colorectal cancer metastatic to the liver: preclinical evaluation in comparison to standard chemotherapy and initial results of a phase I clinical study. Clin Cancer Res 1999 Oct;5(10 Suppl):3232s-3242s.

(74) Witzig TE, Gordon LI, Cabanillas F, Czuczman MS, Emmanouilides C, Joyce R, et al. Randomized controlled trial of yttrium-90-labeled ibritumomab tiuxetan radioimmunotherapy versus rituximab immunotherapy for patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma. J Clin Oncol 2002 May 15;20(10):2453-2463.

(75) Johnson PW, Rohatiner AZ, Whelan JS, Price CG, Love S, Lim J, et al. Patterns of survival in patients with recurrent follicular lymphoma: a 20-year study from a single center. J Clin Oncol 1995 Jan;13(1):140-147.

(76) Tsang RW, Gospodarowicz MK. Radiation therapy for localized low-grade non-Hodgkin's lymphomas. Hematol Oncol 2005 Mar;23(1):10-17.

(77) Horning SJ. Natural history of and therapy for the indolent non-Hodgkin's lymphomas. Semin Oncol 1993 Oct;20(5 Suppl 5):75-88.

(78) Hunault-Berger M, Ifrah N, Solal-Celigny P. Intensive therapies in follicular non-Hodgkin lymphomas. Blood 2002 Aug 15;100(4):1141-1152.

(79) Adachi S, Leoni LM, Carson DA, Nakahata T. Apoptosis induced by molecular targeting therapy in hematological malignancies. Acta Haematol 2004;111(1-2):107-123.

(80) McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. J Clin Oncol 1998 Aug;16(8):2825-2833.

(81) Witzig TE, Flinn IW, Gordon LI, Emmanouilides C, Czuczman MS, Saleh MN, et al. Treatment with ibritumomab tiuxetan radioimmunotherapy in patients with rituximab-refractory follicular non-Hodgkin's lymphoma. J Clin Oncol 2002 Aug 1;20(15):3262-3269.

(82) Kaminski MS, Estes J, Zasadny KR, Francis IR, Ross CW, Tuck M, et al. Radioimmunotherapy with iodine (131)I tositumomab for relapsed or refractory B-cell non-Hodgkin lymphoma: updated results and long-term follow-up of the University of Michigan experience. Blood 2000 Aug 15;96(4):1259-1266.

(83) Kaminski MS, Tuck M, Estes J, Kolstad A, Ross CW, Zasadny K, et al. 131I-tositumomab therapy as initial treatment for follicular lymphoma. N Engl J Med 2005 Feb 3;352(5):441-449.

(84) Kaminski MS, Zelenetz AD, Press OW, Saleh M, Leonard J, Fehrenbacher L, et al. Pivotal study of iodine I 131 tositumomab for chemotherapy-refractory low-grade or transformed low-grade B-cell non-Hodgkin's lymphomas. J Clin Oncol 2001 Oct 1;19(19):3918-3928.

(85) Bennett JM, Kaminski MS, Leonard JP, Vose JM, Zelenetz AD, Knox SJ, et al. Assessment of treatment-related myelodysplastic syndromes and acute myeloid leukemia in patients with non-Hodgkin lymphoma treated with tositumomab and iodine I131 tositumomab. Blood 2005 Jun 15;105(12):4576-4582.

(86) Wiseman GA, Kornmehl E, Leigh B, Erwin WD, Podoloff DA, Spies S, et al. Radiation dosimetry results and safety correlations from 90Y-ibritumomab tiuxetan radioimmunotherapy for relapsed or refractory non-Hodgkin's lymphoma: combined data from 4 clinical trials. J Nucl Med 2003 Mar;44(3):465-474.

(87) Emmanouilides C, Witzig TE, Gordon LI, Vo K, Wiseman GA, Flinn IW, et al. Treatment with yttrium 90 ibritumomab tiuxetan at early relapse is safe and effective in patients with previously treated B-cell non-Hodgkin's lymphoma. Leuk Lymphoma 2006 Apr;47(4):629-636.

(88) Witzig TE, White CA, Gordon LI, Wiseman GA, Emmanouilides C, Murray JL, et al. Safety of yttrium-90 ibritumomab tiuxetan radioimmunotherapy for relapsed low-grade, follicular, or transformed non-hodgkin's lymphoma. J Clin Oncol 2003 Apr 1;21(7):1263-1270.

(89) Emmanouilides C, Witzig TE, White CA, Gordon LI, Wiseman GA, Murray JL, et al. Rapid immune reconstitution after Zevalin is associated with low infectious risk., 3 ed 2002. p. 346-347.

(90) Czuczman MS, Emmanouilides C, Darif M, Witzig TE, Gordon LI, Revell S, et al. Treatment-related myelodysplastic syndrome and acute myelogenous leukemia in patients treated with ibritumomab tiuxetan radioimmunotherapy. J Clin Oncol 2007 Sep 20;25(27):4285-4292.

(91) Emmanouilides C, Witzig TE, Wiseman GA, Gordon LI, Wang H, Schilder R, et al. Safety and efficacy of yttrium-90 ibritumomab tiuxetan in older patients with non-Hodgkin's lymphoma. Cancer Biother Radiopharm 2007 Oct;22(5):684-691.

(92) Ansell SM, Ristow KM, Habermann TM, Wiseman GA, Witzig TE. Subsequent chemotherapy regimens are well tolerated after radioimmunotherapy with yttrium-90 ibritumomab tiuxetan for non-Hodgkin's lymphoma. J Clin Oncol 2002 Sep 15;20(18):3885-3890.

(93) Koppe MJ, Bleichrodt RP, Oyen WJ, Boerman OC. Radioimmunotherapy and colorectal cancer. Br J Surg 2005 Mar;92(3):264-276.

(94) Lane DM, Eagle KF, Begent RH, Hope-Stone LD, Green AJ, Casey JL, et al. Radioimmunotherapy of metastatic colorectal tumours with iodine-131-labelled antibody to carcinoembryonic antigen: phase I/II study with comparative biodistribution of intact and F(ab')2 antibodies. Br J Cancer 1994 Sep;70(3):521-525.

(95) Padera TP, Stoll BR, Tooredman JB, Capen D, di TE, Jain RK. Pathology: cancer cells compress intratumour vessels. Nature 2004 Feb 19;427(6976):695.

(96) Itzkowitz SH, Shi ZR, Kim YS. Heterogeneous expression of two oncodevelopmental antigens, CEA and SSEA-1, in colorectal cancer. Histochem J 1986 Apr;18(4):155-163.

(97) Kaanders JH, Bussink J, van der Kogel AJ. Clinical studies of hypoxia modification in radiotherapy. Semin Radiat Oncol 2004 Jul;14(3):233-240.

(98) Sharkey RM, Goldenberg DM. Perspectives on cancer therapy with radiolabeled monoclonal antibodies. J Nucl Med 2005 Jan;46 Suppl 1:115S-127S.

(99) Jhanwar YS, Divgi C. Current status of therapy of solid tumors. J Nucl Med 2005 Jan;46 Suppl 1:141S-150S.

(100) Goldstein MJ, Mitchell EP. Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. Cancer Invest 2005;23(4):338-351.

(101) Behr TM, Sharkey RM, Juweid MI, Dunn RM, Ying Z, Zhang CH, et al. Factors influencing the pharmacokinetics, dosimetry, and diagnostic accuracy of radioimmunodetection and radioimmunotherapy of carcinoembryonic antigen-expressing tumors. Cancer Res 1996 Apr 15;56(8):1805-1816.

(102) Kraeber-Bodere F, Bardet S, Hoefnagel CA, Vieira MR, Vuillez JP, Murat A, et al. Radioimmunotherapy in medullary thyroid cancer using bispecific antibody and iodine 131-labeled bivalent hapten: preliminary results of a phase I/II clinical trial. Clin Cancer Res 1999 Oct;5(10 Suppl):3190s-3198s.

(103) Begent RH, Ledermann JA, Green AJ, Bagshawe KD, Riggs SJ, Searle F, et al. Antibody distribution and dosimetry in patients receiving radiolabelled antibody therapy for colorectal cancer. Br J Cancer 1989 Sep;60(3):406-412.

(104) Behr TM, Sharkey RM, Juweid ME, Dunn RM, Vagg RC, Ying Z, et al. Phase I/II clinical radioimmunotherapy with an iodine-131-labeled anti-

carcinoembryonic antigen murine monoclonal antibody IgG. J Nucl Med 1997 Jun;38(6):858-870.

(105) Juweid ME, Sharkey RM, Behr T, Swayne LC, Dunn R, Siegel J, et al. Radioimmunotherapy of patients with small-volume tumors using iodine-131-labeled anti-CEA monoclonal antibody NP-4 F(ab')2. J Nucl Med 1996 Sep;37(9):1504-1510.

(106) Meredith RF, Khazaeli MB, Plott WE, Grizzle WE, Liu T, Schlom J, et al. Phase II study of dual 131I-labeled monoclonal antibody therapy with interferon in patients with metastatic colorectal cancer. Clin Cancer Res 1996 Nov;2(11):1811-1818.

(107) Mittal BB, Zimmer MA, Sathiaseelan V, Benson AB, III, Mittal RR, Dutta S, et al. Phase I/II trial of combined 131I anti-CEA monoclonal antibody and hyperthermia in patients with advanced colorectal adenocarcinoma. Cancer 1996 Nov 1;78(9):1861-1870.

(108) Ychou M, Pelegrin A, Faurous P, Robert B, Saccavini JC, Guerreau D, et al. Phase-I/II radio-immunotherapy study with Iodine-131-labeled anti-CEA monoclonal antibody F6 F(ab')2 in patients with non-resectable liver metastases from colorectal cancer. Int J Cancer 1998 Feb 9;75(4):615-619.

(109) Juweid M, Sharkey RM, Swayne LC, Griffiths GL, Dunn R, Goldenberg DM. Pharmacokinetics, dosimetry and toxicity of rhenium-188-labeled anti-carcinoembryonic antigen monoclonal antibody, MN-14, in gastrointestinal cancer. J Nucl Med 1998 Jan;39(1):34-42.

(110) Wong JYC, Chu DZ, Yamauchi DM, Williams LE, Liu A, Wilczynski S, et al. A phase I radioimmunotherapy trial evaluating 90yttrium-labeled anti-carcinoembryonic antigen (CEA) chimeric T84.66 in patients with metastatic CEA-producing malignancies. Clin Cancer Res 2000 Oct;6(10):3855-3863.

(111) Wong JY, Shibata S, Williams LE, Kwok CS, Liu A, Chu DZ, et al. A Phase I trial of 90Y-anti-carcinoembryonic antigen chimeric T84.66 radioimmunotherapy with 5-fluorouracil in patients with metastatic colorectal cancer. Clin Cancer Res 2003 Dec 1;9(16 Pt 1):5842-5852.

(112) Hajjar G, Sharkey RM, Burton J, Zhang CH, Yeldell D, Matthies A, et al. Phase I radioimmunotherapy trial with iodine-131--labeled humanized MN-14 anti-carcinoembryonic antigen monoclonal antibody in patients

with metastatic gastrointestinal and colorectal cancer. Clin Colorectal Cancer 2002 May;2(1):31-42.

(113) Williams LE, Duda RB, Proffitt RT, Beatty BG, Beatty JD, Wong JY, et al. Tumor uptake as a function of tumor mass: a mathematic model. J Nucl Med 1988 Jan;29(1):103-109.

(114) Mayer A, Tsiompanou E, Flynn AA, Pedley RB, Dearling J, Boden R, et al. Higher dose and dose-rate in smaller tumors result in improved tumor control. Cancer Invest 2003 Jun;21(3):382-388.

(115) Fidarova EF, El-Emir E, Boxer GM, Qureshi U, Dearling JL, Robson MP, et al. Microdistribution of targeted, fluorescently labeled anti-carcinoembryonic antigen antibody in metastatic colorectal cancer: implications for radioimmunotherapy. Clin Cancer Res 2008 May 1;14(9):2639-2646.

(116) Behr TM, Liersch T, Greiner-Bechert L, Griesinger F, Behe M, Markus PM, et al. Radioimmunotherapy of small-volume disease of metastatic colorectal cancer. Cancer 2002 Feb 15;94(4 Suppl):1373-1381.

(117) Liersch T, Meller J, Bittrich M, Kulle B, Becker H, Goldenberg DM. Update of carcinoembryonic antigen radioimmunotherapy with (131)I-labetuzumab after salvage resection of colorectal liver metastases: comparison of outcome to a contemporaneous control group. Ann Surg Oncol 2007 Sep;14(9):2577-2590.

(118) Ychou M, Azria D, Menkarios C, Faurous P, Quenet F, Saint-Aubert B, et al. Adjuvant radioimmunotherapy trial with iodine-131-labeled anti-carcinoembryonic antigen monoclonal antibody F6 F(ab')2 after resection of liver metastases from colorectal cancer. Clin Cancer Res 2008 Jun 1;14(11):3487-3493.

(119) Thorpe PE. Vascular targeting agents as cancer therapeutics. Clin Cancer Res 2004 Jan 15;10(2):415-427.

(120) Konerding MA, Malkusch W, Klapthor B, van AC, Fait E, Hill SA, et al. Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. Br J Cancer 1999 May;80(5-6):724-732.

(121) Tozer GM, meer-Beg SM, Baker J, Barber PR, Hill SA, Hodgkiss RJ, et al. Intravital imaging of tumour vascular networks using multi-photon

fluorescence microscopy. Adv Drug Deliv Rev 2005 Jan 2;57(1):135-152.

(122) Amir E, Hughes S, Blackhall F, Thatcher N, Ostoros G, Timar J, et al. Targeting blood vessels for the treatment of non-small cell lung cancer. Curr Cancer Drug Targets 2008 Aug;8(5):392-403.

(123) Pedley RB, Hill SA, Boxer GM, Flynn AA, Boden R, Watson R, et al. Eradication of colorectal xenografts by combined radioimmunotherapy and combretastatin a-4 3-O-phosphate. Cancer Res 2001 Jun 15;61(12):4716-4722.

(124) Pedley RB, El-Emir E, Flynn AA, Boxer GM, Dearling J, Raleigh JA, et al. Synergy between vascular targeting agents and antibody-directed therapy. Int J Radiat Oncol Biol Phys 2002 Dec 1;54(5):1524-1531.

(125) Padhani AR. Dynamic contrast-enhanced MRI in clinical oncology: current status and future directions. J Magn Reson Imaging 2002 Oct;16(4):407-422.

(126) Knopp MV, Weiss E, Sinn HP, Mattern J, Junkermann H, Radeleff J, et al. Pathophysiologic basis of contrast enhancement in breast tumors. J Magn Reson Imaging 1999 Sep;10(3):260-266.

(127) Anderson H, Price P. Clinical measurement of blood flow in tumours using positron emission tomography: a review. Nucl Med Commun 2002 Feb;23(2):131-138.

(128) Raichle ME, Martin WR, Herscovitch P, Mintun MA, Markham J. Brain blood flow measured with intravenous H2(15)O. II. Implementation and validation. J Nucl Med 1983 Sep;24(9):790-798.

(129) Mineura K, Sasajima T, Itoh Y, Sasajima H, Kowada M, Tomura N, et al. Blood flow and metabolism of central neurocytoma: a positron emission tomography study. Cancer 1995 Oct 1;76(7):1224-1232.

(130) Wilson CB, Lammertsma AA, McKenzie CG, Sikora K, Jones T. Measurements of blood flow and exchanging water space in breast tumors using positron emission tomography: a rapid and noninvasive dynamic method. Cancer Res 1992 Mar 15;52(6):1592-1597.

(131) Kubo S, Yamamoto K, Magata Y, Iwasaki Y, Tamaki N, Yonekura Y, et al. Assessment of pancreatic blood flow with positron emission

tomography and oxygen-15 water. Ann Nucl Med 1991 Nov;5(4):133-138.

(132) Yamaguchi A, Taniguchi H, Kunishima S, Koh T, Yamagishi H. Correlation between angiographically assessed vascularity and blood flow in hepatic metastases in patients with colorectal carcinoma. Cancer 2000 Sep 15;89(6):1236-1244.

(133) Anderson HL, Yap JT, Miller MP, Robbins A, Jones T, Price PM. Assessment of pharmacodynamic vascular response in a phase I trial of combretastatin A4 phosphate. J Clin Oncol 2003 Aug 1;21(15):2823-2830.

(134) Nagengast WB, Lub-de Hooge MN, Oosting SF, den Dunnen WF, Warnders FJ, Brouwers AH, et al. VEGF-PET imaging is a noninvasive biomarker showing differential changes in the tumor during sunitinib treatment. Cancer Res 2011 Jan 1;71(1):143-153.

(135) Frackowiak RS, Jones T, Lenzi GL, Heather JD. Regional cerebral oxygen utilization and blood flow in normal man using oxygen-15 and positron emission tomography. Acta Neurol Scand 1980 Dec;62(6):336-344.

(136) Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 1989 Dec 1;49(23):6449-6465.

(137) Semenza GL. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol Med 2001 Aug;7(8):345-350.

(138) Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, et al. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 2000 Aug;157(2):411-421.

(139) Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003 Dec 6;362(9399):1907-1917.

(140) Wang B, Xu H, Gao ZQ, Ning HF, Sun YQ, Cao GW. Increased expression of vascular endothelial growth factor in hepatocellular carcinoma after transcatheter arterial chemoembolization. Acta Radiol 2008 Jun;49(5):523-529.

(141) Virmani S, Rhee TK, Ryu RK, Sato KT, Lewandowski RJ, Mulcahy MF, et al. Comparison of hypoxia-inducible factor-1alpha expression before and after transcatheter arterial embolization in rabbit VX2 liver tumors. J Vasc Interv Radiol 2008 Oct;19(10):1483-1489.

(142) Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. Hepatology 2003 Feb;37(2):429-442.

(143) Pang RW, Joh JW, Johnson PJ, Monden M, Pawlik TM, Poon RT. Biology of hepatocellular carcinoma. Ann Surg Oncol 2008 Apr;15(4):962-971.

(144) Hopfner M, Schuppan D, Scherubl H. Growth factor receptors and related signalling pathways as targets for novel treatment strategies of hepatocellular cancer. World J Gastroenterol 2008 Jan 7;14(1):1-14.

(145) Ribatti D, Vacca A, Nico B, Sansonno D, Dammacco F. Angiogenesis and anti-angiogenesis in hepatocellular carcinoma. Cancer Treat Rev 2006 Oct;32(6):437-444.

(146) Tanaka H, Yamamoto M, Hashimoto N, Miyakoshi M, Tamakawa S, Yoshie M, et al. Hypoxia-independent overexpression of hypoxia-inducible factor 1alpha as an early change in mouse hepatocarcinogenesis. Cancer Res 2006 Dec 1;66(23):11263-11270.

(147) Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 2008 Aug;8(8):618-631.

(148) Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005 Dec;5(12):953-964.

(149) Lewis C, Murdoch C. Macrophage responses to hypoxia: implications for tumor progression and anti-cancer therapies. Am J Pathol 2005 Sep;167(3):627-635.

(150) Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. Immunol Today 1992 Jul;13(7):265-270.

(151) Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 2005 Mar;7(3):211-217.

(152) Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 2002 Mar;196(3):254-265.

(153) Saccani A, Schioppa T, Porta C, Biswas SK, Nebuloni M, Vago L, et al. p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. Cancer Res 2006 Dec 1;66(23):11432-11440.

(154) Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002 Nov;23(11):549-555.

(155) Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006 Jan 27;124(2):263-266.

(156) Chen JJ, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. J Clin Oncol 2005 Feb 10;23(5):953-964.

(157) Luo Y, Zhou H, Krueger J, Kaplan C, Lee SH, Dolman C, et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. J Clin Invest 2006 Aug;116(8):2132-2141.

(158) Venneri MA, De PM, Ponzoni M, Pucci F, Scielzo C, Zonari E, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood 2007 Jun 15;109(12):5276-5285.

(159) De PM, Venneri MA, Galli R, Sergi SL, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 2005 Sep;8(3):211-226.

(160) De PM, Naldini L. Tie2-expressing monocytes (TEMs): novel targets and vehicles of anticancer therapy? Biochim Biophys Acta 2009 Aug;1796(1):5-10.

(161) Murdoch C, Tazzyman S, Webster S, Lewis CE. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. J Immunol 2007 Jun 1;178(11):7405-7411.

(162) Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood 2004 Jun 1;103(11):4150-4156.

(163) De PM, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 2003 Jun;9(6):789-795.

(164) Jeong JO, Kim MO, Kim H, Lee MY, Kim SW, Ii M, et al. Dual angiogenic and neurotrophic effects of bone marrow-derived endothelial progenitor cells on diabetic neuropathy. Circulation 2009 Feb 10;119(5):699-708.

(165) Ribatti D. The paracrine role of Tie-2-expressing monocytes in tumor angiogenesis. Stem Cells Dev 2009 Jun;18(5):703-706.

(166) Figueroa-Vega N, Diaz A, Adrados M, varez-Escola C, Paniagua A, Aragones J, et al. The association of the angiopoietin/Tie-2 system with the development of metastasis and leukocyte migration in neuroendocrine tumors. Endocr Relat Cancer 2010;17(4):897-908.

(167) De PM, Mazzieri R, Politi LS, Pucci F, Zonari E, Sitia G, et al. Tumor-targeted interferon-alpha delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis. Cancer Cell 2008 Oct 7;14(4):299-311.

(168) Lee CY, Tien HF, Hu CY, Chou WC, Lin LI. Marrow angiogenesis-associated factors as prognostic biomarkers in patients with acute myelogenous leukaemia. Br J Cancer 2007 Oct 8;97(7):877-882.

(169) Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001 Jun 19;103(24):2885-2890.

(170) Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999 Aug 6;85(3):221-228.

(171) Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997 Feb 14;275(5302):964-967.

(172) Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 2001 Jun 1;97(11):3658-3661.

(173) Beaudry P, Force J, Naumov GN, Wang A, Baker CH, Ryan A, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. Clin Cancer Res 2005 May 1;11(9):3514-3522.

(174) Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000 Jan;105(1):71-77.

(175) Pathak AP, Hochfeld WE, Goodman SL, Pepper MS. Circulating and imaging markers for angiogenesis. Angiogenesis 2008;11(4):321-335.

(176) Dome B, Dobos J, Tovari J, Paku S, Kovacs G, Ostoros G, et al. Circulating bone marrow-derived endothelial progenitor cells: characterization, mobilization, and therapeutic considerations in malignant disease. Cytometry A 2008 Mar;73(3):186-193.

(177) Elshal MF, Khan SS, Takahashi Y, Solomon MA, McCoy JP, Jr. CD146 (Mel-CAM), an adhesion marker of endothelial cells, is a novel marker of lymphocyte subset activation in normal peripheral blood. Blood 2005 Oct 15;106(8):2923-2924.

(178) Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 2006 Nov;6(11):835-845.

(179) Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000 Feb 1;95(3):952-958.

(180) Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, et al. Vascular endothelial growth factor(165) gene transfer augments

circulating endothelial progenitor cells in human subjects. Circ Res 2000 Jun 23;86(12):1198-1202.

(181) Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood 2005 Sep 1;106(5):1525-1531.

(182) Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003 Feb 13;348(7):593-600.

(183) Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. Circ Res 2000 Sep 15;87(6):434-439.

(184) Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007 Mar 1;109(5):1801-1809.

(185) Monestiroli S, Mancuso P, Burlini A, Pruneri G, Dell'Agnola C, Gobbi A, et al. Kinetics and viability of circulating endothelial cells as surrogate angiogenesis marker in an animal model of human lymphoma. Cancer Res 2001 Jun 1;61(11):4341-4344.

(186) Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis; Implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 2005 Jan;7(1):101-111.

(187) Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, et al. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. Development 2006 Apr;133(8):1543-1551.

(188) Ding YT, Kumar S, Yu DC. The role of endothelial progenitor cells in tumour vasculogenesis. Pathobiology 2008;75(5):265-273.

(189) Sangai T, Ishii G, Kodama K, Miyamoto S, Aoyagi Y, Ito T, et al. Effect of differences in cancer cells and tumor growth sites on recruiting bone marrow-derived endothelial cells and myofibroblasts in cancer-induced stroma. Int J Cancer 2005 Jul 20;115(6):885-892.

(190) Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, et al. Endothelial cells of hematopoietic origin make a significant

contribution to adult blood vessel formation. Circ Res 2000 Oct 27;87(9):728-730.

(191) Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 2003 May 16;300(5622):1155-1159.

(192) Duda DG, Cohen KS, Kozin SV, Perentes JY, Fukumura D, Scadden DT, et al. Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. Blood 2006 Apr 1;107(7):2774-2776.

(193) Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, et al. Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. J Exp Med 2003 Jun 16;197(12):1755-1765.

(194) Yu D, Sun X, Qiu Y, Zhou J, Wu Y, Zhuang L, et al. Identification and clinical significance of mobilized endothelial progenitor cells in tumor vasculogenesis of hepatocellular carcinoma. Clin Cancer Res 2007 Jul 1;13(13):3814-3824.

(195) Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, et al. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci U S A 2008 May 6;105(18):6620-6625.

(196) Kerbel RS, Benezra R, Lyden DC, Hattori K, Heissig B, Nolan DJ, et al. Endothelial progenitor cells are cellular hubs essential for neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition but not adenomas. Proc Natl Acad Sci U S A 2008 Aug 26;105(34):E54.

(197) Rajantie I, Ilmonen M, Alminaite A, Ozerdem U, Alitalo K, Salven P. Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood 2004 Oct 1;104(7):2084-2086.

(198) Gothert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, et al. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. Blood 2004 Sep 15;104(6):1769-1777.

(199) Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001 Nov;7(11):1194-1201.

(200) Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R. Chemokines direct endothelial progenitors into tumor neovessels. Proc Natl Acad Sci U S A 2005 Dec 13;102(50):18111-18116.

(201) Stoll BR, Migliorini C, Kadambi A, Munn LL, Jain RK. A mathematical model of the contribution of endothelial progenitor cells to angiogenesis in tumors: implications for antiangiogenic therapy. Blood 2003 Oct 1;102(7):2555-2561.

(202) Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006 Sep 22;313(5794):1785-1787.

(203) Shaked Y, Tang T, Woloszynek J, Daenen LG, Man S, Xu P, et al. Contribution of granulocyte colony-stimulating factor to the acute mobilization of endothelial precursor cells by vascular disrupting agents. Cancer Res 2009 Oct 1;69(19):7524-7528.

(204) Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De MM, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001 May;7(5):575-583.

(205) Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S, et al. Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. Genes Dev 2007 Jun 15;21(12):1546-1558.

(206) Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, et al. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 2008 Mar;13(3):206-220.

(207) Miyata T, Iizasa H, Sai Y, Fujii J, Terasaki T, Nakashima E. Platelet-derived growth factor-BB (PDGF-BB) induces differentiation of bone marrow endothelial progenitor cell-derived cell line TR-BME2 into mural cells, and changes the phenotype. J Cell Physiol 2005 Sep;204(3):948-955.

(208) Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002 May 31;109(5):625-637.

(209) Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999 Jul 15;18(14):3964-3972.

(210) Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkoviz R, et al. Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. Blood 2001 May 15;97(10):3283-3291.

(211) Bahlmann FH, de GK, Spandau JM, Landry AL, Hertel B, Duckert T, et al. Erythropoietin regulates endothelial progenitor cells. Blood 2004 Feb 1;103(3):921-926.

(212) Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 2003 Aug 15;102(4):1340-1346.

(213) Ho JW, Pang RW, Lau C, Sun CK, Yu WC, Fan ST, et al. Significance of circulating endothelial progenitor cells in hepatocellular carcinoma. Hepatology 2006 Oct;44(4):836-843.

(214) Goon PK, Lip GY, Boos CJ, Stonelake PS, Blann AD. Circulating endothelial cells, endothelial progenitor cells, and endothelial microparticles in cancer. Neoplasia 2006 Feb;8(2):79-88.

(215) Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005 Mar;11(3):261-262.

(216) Dome B, Timar J, Dobos J, Meszaros L, Raso E, Paku S, et al. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. Cancer Res 2006 Jul 15;66(14):7341-7347.

(217) Sussman LK, Upalakalin JN, Roberts MJ, Kocher O, Benjamin LE. Blood markers for vasculogenesis increase with tumor progression in patients with breast carcinoma. Cancer Biol Ther 2003 May;2(3):255-256.

(218) Naik RP, Jin D, Chuang E, Gold EG, Tousimis EA, Moore AL, et al. Circulating endothelial progenitor cells correlate to stage in patients with invasive breast cancer. Breast Cancer Res Treat 2008 Jan;107(1):133-138.

(219) Willett CG, Boucher Y, di TE, Duda DG, Munn LL, Tong RT, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 2004 Feb;10(2):145-147.

(220) Igreja C, Courinha M, Cachaco AS, Pereira T, Cabecadas J, da Silva MG, et al. Characterization and clinical relevance of circulating and biopsy-derived endothelial progenitor cells in lymphoma patients. Haematologica 2007 Apr;92(4):469-477.

(221) Zhang H, Vakil V, Braunstein M, Smith EL, Maroney J, Chen L, et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. Blood 2005 Apr 15;105(8):3286-3294.

(222) Wierzbowska A, Robak T, Krawczynska A, Wrzesien-Kus A, Pluta A, Cebula B, et al. Circulating endothelial cells in patients with acute myeloid leukemia. Eur J Haematol 2005 Dec;75(6):492-497.

(223) Johnston JA, Bacon CM, Riedy MC, O'Shea JJ. Signaling by IL-2 and related cytokines: JAKs, STATs, and relationship to immunodeficiency. J Leukoc Biol 1996 Oct;60(4):441-452.

(224) Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. Proc Natl Acad Sci U S A 1997 Apr 1;94(7):3168-3171.

(225) Waldmann TA, Leonard WJ, Depper JM, Kronke M, Goldman CK, Oh T, et al. Interleukin-2 receptor expression in retrovirus associated adult T-cell leukemia. Princess Takamatsu Symp 1984;15:259-268.

(226) Sheibani K, Winberg CD, van d, V, Blayney DW, Rappaport H. Distribution of lymphocytes with interleukin-2 receptors (TAC antigens) in reactive lymphoproliferative processes, Hodgkin's disease, and non-Hodgkin's lymphomas. An immunohistologic study of 300 cases. Am J Pathol 1987 Apr;127(1):27-37.

(227) Strauchen JA, Breakstone BA. IL-2 receptor expression in human lymphoid lesions. Immunohistochemical study of 166 cases. Am J Pathol 1987 Mar;126(3):506-512.

(228) Waldmann TA, Pastan IH, Gansow OA, Junghans RP. The multichain interleukin-2 receptor: a target for immunotherapy. Ann Intern Med 1992 Jan 15;116(2):148-160.

(229) Kuppers R, Rajewsky K, Zhao M, Simons G, Laumann R, Fischer R, et al. Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. Proc Natl Acad Sci U S A 1994 Nov 8;91(23):10962-10966.

(230) Majhail NS, Weisdorf DJ, DeFor TE, Miller JS, McGlave PB, Slungaard A, et al. Long-term results of autologous stem cell transplantation for primary refractory or relapsed Hodgkin's lymphoma. Biol Blood Marrow Transplant 2006 Oct;12(10):1065-1072.

(231) Freytes CO, Loberiza FR, Rizzo JD, Bashey A, Bredeson CN, Cairo MS, et al. Myeloablative allogeneic hematopoietic stem cell transplantation in patients who experience relapse after autologous stem cell transplantation for lymphoma: a report of the International Bone Marrow Transplant Registry. Blood 2004 Dec 1;104(12):3797-3803.

(232) Lenhard RE, Jr., Order SE, Spunberg JJ, Asbell SO, Leibel SA. Isotopic immunoglobulin: a new systemic therapy for advanced Hodgkin's disease. J Clin Oncol 1985 Oct;3(10):1296-1300.

(233) Bartlett NL, Younes A, Carabasi MH, Forero A, Rosenblatt JD, Leonard JP, et al. A phase 1 multidose study of SGN-30 immunotherapy in patients with refractory or recurrent CD30+ hematologic malignancies. Blood 2008 Feb 15;111(4):1848-1854.

(234) Ansell SM, Horwitz SM, Engert A, Khan KD, Lin T, Strair R, et al. Phase I/II study of an anti-CD30 monoclonal antibody (MDX-060) in Hodgkin's lymphoma and anaplastic large-cell lymphoma. J Clin Oncol 2007 Jul 1;25(19):2764-2769.

(235) Francisco JA, Cerveny CG, Meyer DL, Mixan BJ, Klussman K, Chace DF, et al. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. Blood 2003 Aug 15;102(4):1458-1465.

(236) Oflazoglu E, Kissler KM, Sievers EL, Grewal IS, Gerber HP. Combination of the anti-CD30-auristatin-E antibody-drug conjugate (SGN-35) with chemotherapy improves antitumour activity in Hodgkin lymphoma. Br J Haematol 2008 Jul;142(1):69-73.

(237) Schnell R, Dietlein M, Staak JO, Borchmann P, Schomaecker K, Fischer T, et al. Treatment of refractory Hodgkin's lymphoma patients with an iodine-131-labeled murine anti-CD30 monoclonal antibody. J Clin Oncol 2005 Jul 20;23(21):4669-4678.

(238) Rizvi MA, Evens AM, Tallman MS, Nelson BP, Rosen ST. T-cell non-Hodgkin lymphoma. Blood 2006 Feb 15;107(4):1255-1264.

(239) Rudiger T, Weisenburger DD, Anderson JR, Armitage JO, Diebold J, MacLennan KA, et al. Peripheral T-cell lymphoma (excluding anaplastic large-cell lymphoma): results from the Non-Hodgkin's Lymphoma Classification Project. Ann Oncol 2002 Jan;13(1):140-149.

(240) Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, Harrington W, Jr., et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol 2009 Jan 20;27(3):453-459.

(241) Matutes E, Taylor GP, Cavenagh J, Pagliuca A, Bareford D, Domingo A, et al. Interferon alpha and zidovudine therapy in adult T-cell leukaemia lymphoma: response and outcome in 15 patients. Br J Haematol 2001 Jun;113(3):779-784.

(242) Hermine O, Allard I, Levy V, Arnulf B, Gessain A, Bazarbachi A. A prospective phase II clinical trial with the use of zidovudine and interferon-alpha in the acute and lymphoma forms of adult T-cell leukemia/lymphoma. Hematol J 2002;3(6):276-282.

(243) Waldmann TA, Goldman CK, Bongiovanni KF, Sharrow SO, Davey MP, Cease KB, et al. Therapy of patients with human T-cell lymphotrophic virus I-induced adult T-cell leukemia with anti-Tac, a monoclonal antibody to the receptor for interleukin-2. Blood 1988 Nov;72(5):1805-1816.

(244) Waldmann TA, White JD, Carrasquillo JA, Reynolds JC, Paik CH, Gansow OA, et al. Radioimmunotherapy of interleukin-2R alpha-expressing adult T-cell leukemia with Yttrium-90-labeled anti-Tac. Blood 1995 Dec 1;86(11):4063-4075.

(245) Amlot PL, Rawlings E, Fernando ON, Griffin PJ, Heinrich G, Schreier MH, et al. Prolonged action of a chimeric interleukin-2 receptor (CD25) monoclonal antibody used in cadaveric renal transplantation. Transplantation 1995 Oct 15;60(7):748-756.

(246) Nashan B, Moore R, Amlot P, Schmidt AG, Abeywickrama K, Soulillou JP. Randomised trial of basiliximab versus placebo for control of acute cellular rejection in renal allograft recipients. CHIB 201 International Study Group. Lancet 1997 Oct 25;350(9086):1193-1198.

(247) Adam T. Radioiodination for therapy. Ann Clin Biochem 1989 May;26 (Pt 3):244-245.

(248) Juweid ME, Stroobants S, Hoekstra OS, Mottaghy FM, Dietlein M, Guermazi A, et al. Use of positron emission tomography for response assessment of lymphoma: consensus of the Imaging Subcommittee of International Harmonization Project in Lymphoma. J Clin Oncol 2007 Feb 10;25(5):571-578.

(249) Cheson BD. The International Harmonization Project for response criteria in lymphoma clinical trials. Hematol Oncol Clin North Am 2007 Oct;21(5):841-854.

(250) de Jong WK, van der Heijden HF, Pruim J, Dalesio O, Oyen WJ, Groen HJ. Prognostic value of different metabolic measurements with fluorine-18 fluorodeoxyglucose positron emission tomography in resectable non-small cell lung cancer: a two-center study. J Thorac Oncol 2007 Nov;2(11):1007-1012.

(251) Green AJ, Francis RJ, Baig S, Begent RH. Semiautomatic volume of interest drawing for (18)F-FDG image analysis-method and preliminary results. Eur J Nucl Med Mol Imaging 2008 Feb;35(2):393-406.

(252) Gause A, Roschansky V, Tschiersch A, Smith K, Hasenclever D, Schmits R, et al. Low serum interleukin-2 receptor levels correlate with a good prognosis in patients with Hodgkin's lymphoma. Ann Oncol 1991 Feb;2 Suppl 2:43-47.

(253) Baechler S, Hobbs RF, Jacene HA, Bochud FO, Wahl RL, Sgouros G. Predicting hematologic toxicity in patients undergoing radioimmunotherapy with 90Y-ibritumomab tiuxetan or 131I-tositumomab. J Nucl Med 2010 Dec;51(12):1878-1884.

(254) Siegel JA, Yeldell D, Goldenberg DM, Stabin MG, Sparks RB, Sharkey RM, et al. Red marrow radiation dose adjustment using plasma FLT3-L cytokine levels: improved correlations between hematologic toxicity and bone marrow dose for radioimmunotherapy patients. J Nucl Med 2003 Jan;44(1):67-76.

(255) Koenecke C, Ukena SN, Ganser A, Franzke A. Regulatory T cells as therapeutic target in Hodgkin's lymphoma. Expert Opin Ther Targets 2008 Jun;12(6):769-782.

(256) Schmitz N, Pfistner B, Sextro M, Sieber M, Carella AM, Haenel M, et al. Aggressive conventional chemotherapy compared with high-dose chemotherapy with autologous haemopoietic stem-cell transplantation for relapsed chemosensitive Hodgkin's disease: a randomised trial. Lancet 2002 Jun 15;359(9323):2065-2071.

(257) Lin CM, Singh SB, Chu PS, Dempcy RO, Schmidt JM, Pettit GR, et al. Interactions of tubulin with potent natural and synthetic analogs of the antimitotic agent combretastatin: a structure-activity study. Mol Pharmacol 1988 Aug;34(2):200-208.

(258) Kanthou C, Tozer GM. The tumor vascular targeting agent combretastatin A-4-phosphate induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells. Blood 2002 Mar 15;99(6):2060-2069.

(259) Galbraith SM, Chaplin DJ, Lee F, Stratford MR, Locke RJ, Vojnovic B, et al. Effects of combretastatin A4 phosphate on endothelial cell morphology in vitro and relationship to tumour vascular targeting activity in vivo. Anticancer Res 2001 Jan;21(1A):93-102.

(260) Vincent L, Kermani P, Young LM, Cheng J, Zhang F, Shido K, et al. Combretastatin A4 phosphate induces rapid regression of tumor neovessels and growth through interference with vascular endothelial-cadherin signaling. J Clin Invest 2005 Nov;115(11):2992-3006.

(261) Dark GG, Hill SA, Prise VE, Tozer GM, Pettit GR, Chaplin DJ. Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. Cancer Res 1997 May 15;57(10):1829-1834.

(262) Tozer GM, Prise VE, Wilson J, Locke RJ, Vojnovic B, Stratford MR, et al. Combretastatin A-4 phosphate as a tumor vascular-targeting agent:

early effects in tumors and normal tissues. Cancer Res 1999 Apr 1;59(7):1626-1634.

(263) Tozer GM, Prise VE, Wilson J, Cemazar M, Shan S, Dewhirst MW, et al. Mechanisms associated with tumor vascular shut-down induced by combretastatin A-4 phosphate: intravital microscopy and measurement of vascular permeability. Cancer Res 2001 Sep 1;61(17):6413-6422.

(264) El-Emir E, Boxer GM, Petrie IA, Boden RW, Dearling JL, Begent RH, et al. Tumour parameters affected by combretastatin A-4 phosphate therapy in a human colorectal xenograft model in nude mice. Eur J Cancer 2005 Mar;41(5):799-806.

(265) Rustin GJ, Galbraith SM, Anderson H, Stratford M, Folkes LK, Sena L, et al. Phase I clinical trial of weekly combretastatin A4 phosphate: clinical and pharmacokinetic results. J Clin Oncol 2003 Aug 1;21(15):2815-2822.

(266) Stevenson JP, Rosen M, Sun W, Gallagher M, Haller DG, Vaughn D, et al. Phase I trial of the antivascular agent combretastatin A4 phosphate on a 5-day schedule to patients with cancer: magnetic resonance imaging evidence for altered tumor blood flow. J Clin Oncol 2003 Dec 1;21(23):4428-4438.

(267) Dowlati A, Robertson K, Cooney M, Petros WP, Stratford M, Jesberger J, et al. A phase I pharmacokinetic and translational study of the novel vascular targeting agent combretastatin a-4 phosphate on a single-dose intravenous schedule in patients with advanced cancer. Cancer Res 2002 Jun 15;62(12):3408-3416.

(268) Pedley RB, Begent RH, Boden JA, Boxer GM, Boden R, Keep PA. Enhancement of radioimmunotherapy by drugs modifying tumour blood flow in a colonic xenograft model. Int J Cancer 1994 Jun 15;57(6):830-835.

(269) Pedley RB, Boden JA, Boden R, Boxer GM, Flynn AA, Keep PA, et al. Ablation of colorectal xenografts with combined radioimmunotherapy and tumor blood flow-modifying agents. Cancer Res 1996 Jul 15;56(14):3293-3300.

(270) Boxer GM, Abassi AM, Pedley RB, Begent RH. Localisation of monoclonal antibodies reacting with different epitopes on

carcinoembryonic antigen (CEA)--implications for targeted therapy. Br J Cancer 1994 Feb;69(2):307-314.

(271) Harwood PJ, Britton DW, Southall PJ, Boxer GM, Rawlins G, Rogers GT. Mapping epitope characteristics on carcinoembryonic antigen. Br J Cancer 1986 Jul;54(1):75-82.

(272) Pedley RB, Boden J, Keep PA, Harwood PJ, Green AJ, Rogers GT. Relationship between tumour size and uptake of radiolabelled anti-CEA in a colon tumour xenograft. Eur J Nucl Med 1987;13(4):197-202.

(273) Tofts PS, Kermode AG. Measurement of the blood-brain barrier permeability and leakage space using dynamic MR imaging. 1. Fundamental concepts. Magn Reson Med 1991 Feb;17(2):357-367.

(274) Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, et al. Combretastatin A4 phosphate has tumor antivascular activity in rat and man as demonstrated by dynamic magnetic resonance imaging. J Clin Oncol 2003 Aug 1;21(15):2831-2842.

(275) Maxwell RJ, Wilson J, Prise VE, Vojnovic B, Rustin GJ, Lodge MA, et al. Evaluation of the anti-vascular effects of combretastatin in rodent tumours by dynamic contrast enhanced MRI. NMR Biomed 2002 Apr;15(2):89-98.

(276) Meyer T, Gaya AM, Dancey G, Stratford MR, Othman S, Sharma SK, et al. A phase I trial of radioimmunotherapy with 131I-A5B7 anti-CEA antibody in combination with combretastatin-A4-phosphate in advanced gastrointestinal carcinomas. Clin Cancer Res 2009 Jul 1;15(13):4484-4492.

(277) Quan H, Xu Y, Lou L. p38 MAPK, but not ERK1/2, is critically involved in the cytotoxicity of the novel vascular disrupting agent combretastatin A4. Int J Cancer 2008 Apr 15;122(8):1730-1737.

(278) Akerley WL, Schabel M, Morrell G, Horrath E, Yu M, Johnsson B, et al. A randomized phase 2 trial of combretatstatin A4 phosphate (CA4P) in combination with paclitaxel and carboplatin to evaluate safety and efficacy in subjects with advanced imageable malignancies. 2007.

(279) Rustin GJ, Shreeves G, Nathan PD, Gaya A, Ganesan TS, Wang D, et al. A Phase Ib trial of CA4P (combretastatin A-4 phosphate), carboplatin,

and paclitaxel in patients with advanced cancer. Br J Cancer 2010 Apr 27;102(9):1355-1360.

(280) Bilenker JH, Flaherty KT, Rosen M, Davis L, Gallagher M, Stevenson JP, et al. Phase I trial of combretastatin a-4 phosphate with carboplatin. Clin Cancer Res 2005 Feb 15;11(4):1527-1533.

(281) Horsman MR, Siemann DW. Pathophysiologic effects of vascular-targeting agents and the implications for combination with conventional therapies. Cancer Res 2006 Dec 15;66(24):11520-11539.

(282) Borsi L, Balza E, Bestagno M, Castellani P, Carnemolla B, Biro A, et al. Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. Int J Cancer 2002 Nov 1;102(1):75-85.

(283) Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005 Dec 15;438(7070):932-936.

(284) Poon RT, Fan ST, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol 2001 Feb 15;19(4):1207-1225.

(285) Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971 Nov 18;285(21):1182-1186.

(286) Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000 Sep 14;407(6801):249-257.

(287) Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005 Feb 10;23(5):1011-1027.

(288) Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983 Feb 25;219(4587):983-985.

(289) Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989 Dec 8;246(4935):1306-1309.

(290) Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989 Dec 8;246(4935):1309-1312.

(291) Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996 Apr 4;380(6573):439-442.

(292) Jubb AM, Hurwitz HI, Bai W, Holmgren EB, Tobin P, Guerrero AS, et al. Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. J Clin Oncol 2006 Jan 10;24(2):217-227.

(293) Smith BD, Smith GL, Carter D, Sasaki CT, Haffty BG. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. J Clin Oncol 2000 May;18(10):2046-2052.

(294) Yamamoto Y, Toi M, Kondo S, Matsumoto T, Suzuki H, Kitamura M, et al. Concentrations of vascular endothelial growth factor in the sera of normal controls and cancer patients. Clin Cancer Res 1996 May;2(5):821-826.

(295) Fuhrmann-Benzakein E, Ma MN, Rubbia-Brandt L, Mentha G, Ruefenacht D, Sappino AP, et al. Elevated levels of angiogenic cytokines in the plasma of cancer patients. Int J Cancer 2000 Jan 1;85(1):40-45.

(296) Hyodo I, Doi T, Endo H, Hosokawa Y, Nishikawa Y, Tanimizu M, et al. Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer 1998 Dec;34(13):2041-2045.

(297) Linder C, Linder S, Munck-Wikland E, Strander H. Independent expression of serum vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in patients with carcinoma and sarcoma. Anticancer Res 1998 May;18(3B):2063-2068.

(298) Kitamura M, Toi M, Arai K, Iwasaki Y, Suzuki H, Matsuo K. Concentrations of vascular endothelial growth factor in the sera of gastric cancer patients. Oncol Rep 1998 Nov;5(6):1419-1424.

(299) Mahner S, Woelber L, Eulenburg C, Schwarz J, Carney W, Jaenicke F, et al. TIMP-1 and VEGF-165 serum concentration during first-line therapy of ovarian cancer patients. BMC Cancer 2010;10:139.

(300) Schoenleber SJ, Kurtz DM, Talwalkar JA, Roberts LR, Gores GJ. Prognostic role of vascular endothelial growth factor in hepatocellular carcinoma: systematic review and meta-analysis. Br J Cancer 2009 May 5;100(9):1385-1392.

(301) Poon RT, Lau CP, Cheung ST, Yu WC, Fan ST. Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma. Cancer Res 2003 Jun 15;63(12):3121-3126.

(302) Salven P, Orpana A, Joensuu H. Leukocytes and platelets of patients with cancer contain high levels of vascular endothelial growth factor. Clin Cancer Res 1999 Mar;5(3):487-491.

(303) George ML, Eccles SA, Tutton MG, Abulafi AM, Swift RI. Correlation of plasma and serum vascular endothelial growth factor levels with platelet count in colorectal cancer: clinical evidence of platelet scavenging? Clin Cancer Res 2000 Aug;6(8):3147-3152.

(304) Yoshiji H, Kuriyama S, Yoshii J, Yamazaki M, Kikukawa M, Tsujinoue H, et al. Vascular endothelial growth factor tightly regulates in vivo development of murine hepatocellular carcinoma cells. Hepatology 1998 Dec;28(6):1489-1496.

(305) Wada H, Nagano H, Yamamoto H, Yang Y, Kondo M, Ota H, et al. Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. Liver Int 2006 May;26(4):414-423.

(306) Moon WS, Rhyu KH, Kang MJ, Lee DG, Yu HC, Yeum JH, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? Mod Pathol 2003 Jun;16(6):552-557.

(307) Li X, Feng GS, Zheng CS, Zhuo CK, Liu X. Expression of plasma vascular endothelial growth factor in patients with hepatocellular carcinoma and effect of transcatheter arterial chemoembolization therapy on plasma vascular endothelial growth factor level. World J Gastroenterol 2004 Oct 1;10(19):2878-2882.

(308) Poon RT, Ho JW, Tong CS, Lau C, Ng IO, Fan ST. Prognostic significance of serum vascular endothelial growth factor and endostatin in patients with hepatocellular carcinoma. Br J Surg 2004 Oct;91(10):1354-1360.

(309) Poon RT, Lau C, Yu WC, Fan ST, Wong J. High serum levels of vascular endothelial growth factor predict poor response to transarterial chemoembolization in hepatocellular carcinoma: a prospective study. Oncol Rep 2004 May;11(5):1077-1084.

(310) Poon RT, Lau C, Pang R, Ng KK, Yuen J, Fan ST. High serum vascular endothelial growth factor levels predict poor prognosis after radiofrequency ablation of hepatocellular carcinoma: importance of tumor biomarker in ablative therapies. Ann Surg Oncol 2007 Jun;14(6):1835-1845.

(311) Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997 Jul 4;277(5322):55-60.

(312) Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG. The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. J Cell Sci 2005 Feb 15;118(Pt 4):771-780.

(313) Procopio WN, Pelavin PI, Lee WM, Yeilding NM. Angiopoietin-1 and -2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. J Biol Chem 1999 Oct 15;274(42):30196-30201.

(314) Benest AV, Augustin HG. Cancer: Blood vessels kept quiet. Nature 2009 Mar 5;458(7234):41-42.

(315) Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996 Dec 27;87(7):1171-1180.

(316) Stoeltzing O, Ahmad SA, Liu W, McCarty MF, Parikh AA, Fan F, et al. Angiopoietin-1 inhibits tumour growth and ascites formation in a murine model of peritoneal carcinomatosis. Br J Cancer 2002 Nov 4;87(10):1182-1187.

(317) Stoeltzing O, Ahmad SA, Liu W, McCarty MF, Wey JS, Parikh AA, et al. Angiopoietin-1 inhibits vascular permeability, angiogenesis, and growth of hepatic colon cancer tumors. Cancer Res 2003 Jun 15;63(12):3370-3377.

(318) Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. Exp Cell Res 2006 Mar 10;312(5):630-641.

(319) Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, et al. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res 1998 Aug 10;83(3):233-240.

(320) Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998 Oct 16;282(5388):468-471.

(321) Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, et al. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell 2002 Sep;3(3):411-423.

(322) Scappaticci FA. Mechanisms and future directions for angiogenesis-based cancer therapies. J Clin Oncol 2002 Sep 15;20(18):3906-3927.

(323) Leach L, Babawale MO, Anderson M, Lammiman M. Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta. J Vasc Res 2002 May;39(3):246-259.

(324) Tait CR, Jones PF. Angiopoietins in tumours: the angiogenic switch. J Pathol 2004 Sep;204(1):1-10.

(325) Bach F, Uddin FJ, Burke D. Angiopoietins in malignancy. Eur J Surg Oncol 2007 Feb;33(1):7-15.

(326) Etoh T, Inoue H, Tanaka S, Barnard GF, Kitano S, Mori M. Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases. Cancer Res 2001 Mar 1;61(5):2145-2153.

(327) Yoshiji H, Kuriyama S, Noguchi R, Yoshii J, Ikenaka Y, Yanase K, et al. Angiopoietin 2 displays a vascular endothelial growth factor dependent synergistic effect in hepatocellular carcinoma development in mice. Gut 2005 Dec;54(12):1768-1775.

(328) Nasarre P, Thomas M, Kruse K, Helfrich I, Wolter V, Deppermann C, et al. Host-derived angiopoietin-2 affects early stages of tumor

development and vessel maturation but is dispensable for later stages of tumor growth. Cancer Res 2009 Feb 15;69(4):1324-1333.

(329) Hayes AJ, Huang WQ, Yu J, Maisonpierre PC, Liu A, Kern FG, et al. Expression and function of angiopoietin-1 in breast cancer. Br J Cancer 2000 Nov;83(9):1154-1160.

(330) Oka N, Yamamoto Y, Takahashi M, Nishitani M, Kanayama HO, Kagawa S. Expression of angiopoietin-1 and -2, and its clinical significance in human bladder cancer. BJU Int 2005 Mar;95(4):660-663.

(331) Tanaka S, Mori M, Sakamoto Y, Makuuchi M, Sugimachi K, Wands JR. Biologic significance of angiopoietin-2 expression in human hepatocellular carcinoma. J Clin Invest 1999 Feb;103(3):341-345.

(332) Hata K, Udagawa J, Fujiwaki R, Nakayama K, Otani H, Miyazaki K. Expression of angiopoietin-1, angiopoietin-2, and Tie2 genes in normal ovary with corpus luteum and in ovarian cancer. Oncology 2002;62(4):340-348.

(333) Ahmad SA, Liu W, Jung YD, Fan F, Reinmuth N, Bucana CD, et al. Differential expression of angiopoietin-1 and angiopoietin-2 in colon carcinoma. A possible mechanism for the initiation of angiogenesis. Cancer 2001 Sep 1;92(5):1138-1143.

(334) Mitsuhashi N, Shimizu H, Ohtsuka M, Wakabayashi Y, Ito H, Kimura F, et al. Angiopoietins and Tie-2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. Hepatology 2003 May;37(5):1105-1113.

(335) Wong MP, Chan SY, Fu KH, Leung SY, Cheung N, Yuen ST, et al. The angiopoietins, tie2 and vascular endothelial growth factor are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas. Lung Cancer 2000 Jul;29(1):11-22.

(336) Sallinen H, Heikura T, Laidinen S, Kosma VM, Heinonen S, Yla-Herttuala S, et al. Preoperative angiopoietin-2 serum levels: a marker of malignant potential in ovarian neoplasms and poor prognosis in epithelial ovarian cancer. Int J Gynecol Cancer 2010 Dec;20(9):1498-1505.

(337) Volkova E, Willis JA, Wells JE, Robinson BA, Dachs GU, Currie MJ. Association of angiopoietin-2, C-reactive protein and markers of

obesity and insulin resistance with survival outcome in colorectal cancer. Br J Cancer 2011 Jan 4;104(1):51-59.

(338) Jo MJ, Lee JH, Nam BH, Kook MC, Ryu KW, Choi IJ, et al. Preoperative serum angiopoietin-2 levels correlate with lymph node status in patients with early gastric cancer. Ann Surg Oncol 2009 Jul;16(7):2052-2057.

(339) Kopczynska E, Makarewicz R, Biedka M, Kaczmarczyk A, Kardymowicz H, Tyrakowski T. Plasma concentration of angiopoietin-1, angiopoietin-2 and Tie-2 in cervical cancer. Eur J Gynaecol Oncol 2009;30(6):646-649.

(340) Helfrich I, Edler L, Sucker A, Thomas M, Christian S, Schadendorf D, et al. Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. Clin Cancer Res 2009 Feb 15;15(4):1384-1392.

(341) Srirajaskanthan R, Dancey G, Hackshaw A, Luong T, Caplin ME, Meyer T. Circulating angiopoietin-2 is elevated in patients with neuroendocrine tumours and correlates with disease burden and prognosis. Endocr Relat Cancer 2009 Sep;16(3):967-976.

(342) Park JH, Choi H, Kim YB, Kim YS, Sheen SS, Choi JH, et al. Serum angiopoietin-1 as a prognostic marker in resected early stage lung cancer. Lung Cancer 2009 Dec;66(3):359-364.

(343) Goede V, Coutelle O, Neuneier J, Reinacher-Schick A, Schnell R, Koslowsky TC, et al. Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy. Br J Cancer 2010 Oct 26;103(9):1407-1414.

(344) Anargyrou K, Terpos E, Vassilakopoulos TP, Pouli A, Sachanas S, Tzenou T, et al. Normalization of the serum angiopoietin-1 to angiopoietin-2 ratio reflects response in refractory/resistant multiple myeloma patients treated with bortezomib. Haematologica 2008 Mar;93(3):451-454.

(345) Naumnik W, Chyczewska E, Ossolinska M. Serum levels of angiopoietin-1, angiopoietin-2, and their receptor tie-2 in patients with nonsmall cell lung cancer during chemotherapy. Cancer Invest 2009 Aug;27(7):741-746.

(346) Torimura T, Ueno T, Kin M, Harada R, Taniguchi E, Nakamura T, et al. Overexpression of angiopoietin-1 and angiopoietin-2 in hepatocellular carcinoma. J Hepatol 2004 May;40(5):799-807.

(347) Zeng W, Gouw AS, van den Heuvel MC, Zwiers PJ, Zondervan PE, Poppema S, et al. The angiogenic makeup of human hepatocellular carcinoma does not favor vascular endothelial growth factor/angiopoietin-driven sprouting neovascularization. Hepatology 2008 Nov;48(5):1517-1527.

(348) Scholz A, Rehm VA, Rieke S, Derkow K, Schulz P, Neumann K, et al. Angiopoietin-2 serum levels are elevated in patients with liver cirrhosis and hepatocellular carcinoma. Am J Gastroenterol 2007 Nov;102(11):2471-2481.

(349) Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 2005 Apr;16(2):107-137.

(350) El-Assal ON, Yamanoi A, Ono T, Kohno H, Nagasue N. The clinicopathological significance of heparanase and basic fibroblast growth factor expressions in hepatocellular carcinoma. Clin Cancer Res 2001 May;7(5):1299-1305.

(351) Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC, et al. Fibroblast growth factor 2 control of vascular tone. Nat Med 1998 Feb;4(2):201-207.

(352) Bosse Y, Rola-Pleszczynski M. FGF2 in asthmatic airway-smooth-muscle-cell hyperplasia. Trends Mol Med 2008 Jan;14(1):3-11.

(353) Ware JA, Simons M. Angiogenesis in ischemic heart disease. Nat Med 1997 Feb;3(2):158-164.

(354) Asahara T, Bauters C, Zheng LP, Takeshita S, Bunting S, Ferrara N, et al. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 1995 Nov 1;92(9 Suppl):II365-II371.

(355) Grose R, Dickson C. Fibroblast growth factor signaling in tumorigenesis. Cytokine Growth Factor Rev 2005 Apr;16(2):179-186.

(356) Colomer R, Aparicio J, Montero S, Guzman C, Larrodera L, Cortes-Funes H. Low levels of basic fibroblast growth factor (bFGF) are associated with a poor prognosis in human breast carcinoma. Br J Cancer 1997;76(9):1215-1220.

(357) Obermair A, Speiser P, Reisenberger K, Ullrich R, Czerwenka K, Kaider A, et al. Influence of intratumoral basic fibroblast growth factor concentration on survival in ovarian cancer patients. Cancer Lett 1998 Aug 14;130(1-2):69-76.

(358) Pichon MF, Moulin G, Pallud C, Pecking A, Floiras JL. Serum bFGF (basic fibroblast growth factor) and CA 15.3 in the monitoring of breast cancer patients. Anticancer Res 2000 Mar;20(2B):1189-1194.

(359) Brattstrom D, Bergqvist M, Larsson A, Holmertz J, Hesselius P, Rosenberg L, et al. Basic fibroblast growth factor and vascular endothelial growth factor in sera from non-small cell lung cancer patients. Anticancer Res 1998 Mar;18(2A):1123-1127.

(360) Hsu PI, Chow NH, Lai KH, Yang HB, Chan SH, Lin XZ, et al. Implications of serum basic fibroblast growth factor levels in chronic liver diseases and hepatocellular carcinoma. Anticancer Res 1997 Jul;17(4A):2803-2809.

(361) Dietz A, Rudat V, Conradt C, Weidauer H, Ho A, Moehler T. Prognostic relevance of serum levels of the angiogenic peptide bFGF in advanced carcinoma of the head and neck treated by primary radiochemotherapy. Head Neck 2000 Oct;22(7):666-673.

(362) Sliutz G, Tempfer C, Obermair A, Reinthaller A, Gitsch G, Kainz C. Serum evaluation of basic fibroblast growth factor in cervical cancer patients. Cancer Lett 1995 Aug 1;94(2):227-231.

(363) Mise M, Arii S, Higashituji H, Furutani M, Niwano M, Harada T, et al. Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. Hepatology 1996 Mar;23(3):455-464.

(364) Poon RT, Ng IO, Lau C, Yu WC, Fan ST, Wong J. Correlation of serum basic fibroblast growth factor levels with clinicopathologic features and postoperative recurrence in hepatocellular carcinoma. Am J Surg 2001 Sep;182(3):298-304.

(365) Chao Y, Li CP, Chau GY, Chen CP, King KL, Lui WY, et al. Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. Ann Surg Oncol 2003 May;10(4):355-362.

(366) Sasaki R, Masuda S, Nagao M. Erythropoietin: multiple physiological functions and regulation of biosynthesis. Biosci Biotechnol Biochem 2000 Sep;64(9):1775-1793.

(367) Leyland-Jones B. Evidence for erythropoietin as a molecular targeting agent. Semin Oncol 2002 Jun;29(3 Suppl 11):145-154.

(368) Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, et al. Erythropoietin regulates tumour growth of human malignancies. Carcinogenesis 2003 Jun;24(6):1021-1029.

(369) Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev 1992 Apr;72(2):449-489.

(370) Lacombe C, Mayeux P. The molecular biology of erythropoietin. Nephrol Dial Transplant 1999;14 Suppl 2:22-28.

(371) Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 1999 Aug;126(16):3597-3605.

(372) Nakamatsu K, Nishimura Y, Suzuki M, Kanamori S, Maenishi O, Yasuda Y. Erythropoietin/erythropoietin-receptor system as an angiogenic factor in chemically induced murine hepatic tumors. Int J Clin Oncol 2004 Jun;9(3):184-188.

(373) Bianchi R, Buyukakilli B, Brines M, Savino C, Cavaletti G, Oggioni N, et al. Erythropoietin both protects from and reverses experimental diabetic neuropathy. Proc Natl Acad Sci U S A 2004 Jan 20;101(3):823-828.

(374) Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci U S A 1990 Aug;87(15):5978-5982.

(375) Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured

endothelial cells and stimulates neovascularization in vivo. Blood 1999 Apr 15;93(8):2627-2636.

(376) Glaspy J, Bukowski R, Steinberg D, Taylor C, Tchekmedyian S, Vadhan-Raj S. Impact of therapy with epoetin alfa on clinical outcomes in patients with nonmyeloid malignancies during cancer chemotherapy in community oncology practice. Procrit Study Group. J Clin Oncol 1997 Mar;15(3):1218-1234.

(377) Leyland-Jones B, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, et al. Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. J Clin Oncol 2005 Sep 1;23(25):5960-5972.

(378) Henke M, Mattern D, Pepe M, Bezay C, Weissenberger C, Werner M, et al. Do erythropoietin receptors on cancer cells explain unexpected clinical findings? J Clin Oncol 2006 Oct 10;24(29):4708-4713.

(379) Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A. Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. Cancer 2002 Sep 1;95(5):969-981.

(380) Kumar SM, Acs G, Fang D, Herlyn M, Elder DE, Xu X. Functional erythropoietin autocrine loop in melanoma. Am J Pathol 2005 Mar;166(3):823-830.

(381) Acs G, Zhang PJ, McGrath CM, Acs P, McBroom J, Mohyeldin A, et al. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. Am J Pathol 2003 Jun;162(6):1789-1806.

(382) Rosti V, Pedrazzoli P, Ponchio L, Zibera C, Novella A, Lucotti C, et al. Effect of recombinant human erythropoietin on hematopoietic and non-hematopoietic malignant cell growth in vitro. Haematologica 1993 Jul;78(4):208-212.

(383) Westphal G, Niederberger E, Blum C, Wollman Y, Knoch TA, Rebel W, et al. Erythropoietin and G-CSF receptors in human tumor cells: expression and aspects regarding functionality. Tumori 2002 Mar;88(2):150-159.

(384) Selzer E, Wacheck V, Kodym R, Schlagbauer-Wadl H, Schlegel W, Pehamberger H, et al. Erythropoietin receptor expression in human melanoma cells. Melanoma Res 2000 Oct;10(5):421-426.

(385) Berdel WE, Oberberg D, Reufi B, Thiel E. Studies on the role of recombinant human erythropoietin in the growth regulation of human nonhematopoietic tumor cells in vitro. Ann Hematol 1991 Jul;63(1):5-8.

(386) Blackwell KL, Kirkpatrick JP, Snyder SA, Broadwater G, Farrell F, Jolliffe L, et al. Human recombinant erythropoietin significantly improves tumor oxygenation independent of its effects on hemoglobin. Cancer Res 2003 Oct 1;63(19):6162-6165.

(387) Thews O, Koenig R, Kelleher DK, Kutzner J, Vaupel P. Enhanced radiosensitivity in experimental tumours following erythropoietin treatment of chemotherapy-induced anaemia. Br J Cancer 1998 Sep;78(6):752-756.

(388) Thews O, Kelleher DK, Vaupel P. Erythropoietin restores the anemia-induced reduction in cyclophosphamide cytotoxicity in rat tumors. Cancer Res 2001 Feb 15;61(4):1358-1361.

(389) Ribatti D, Marzullo A, Gentile A, Longo V, Nico B, Vacca A, et al. Erythropoietin/erythropoietin-receptor system is involved in angiogenesis in human hepatocellular carcinoma. Histopathology 2007 Apr;50(5):591-596.

(390) Matsuyama M, Yamazaki O, Horii K, Higaki I, Kawai S, Mikami S, et al. Erythrocytosis caused by an erythropoietin-producing hepatocellular carcinoma. J Surg Oncol 2000 Nov;75(3):197-202.

(391) Fisher B, Redmond C, Poisson R, Margolese R, Wolmark N, Wickerham L, et al. Eight-year results of a randomized clinical trial comparing total mastectomy and lumpectomy with or without irradiation in the treatment of breast cancer. N Engl J Med 1989 Mar 30;320(13):822-828.

(392) Kim YB, Park YN, Park C. Increased proliferation activities of vascular endothelial cells and tumour cells in residual hepatocellular carcinoma following transcatheter arterial embolization. Histopathology 2001 Feb;38(2):160-166.

(393) Cabrera R, Pannu DS, Caridi J, Firpi RJ, Soldevila-Pico C, Morelli G, et al. The combination of sorafenib with transarterial chemoembolisation for hepatocellular carcinoma. Aliment Pharmacol Ther 2011 Jul;34(2):205-213.

(394) Yang ZF, Poon RT, To J, Ho DW, Fan ST. The potential role of hypoxia inducible factor 1alpha in tumor progression after hypoxia and chemotherapy in hepatocellular carcinoma. Cancer Res 2004 Aug 1;64(15):5496-5503.

(395) Shim JH, Park JW, Kim JH, An M, Kong SY, Nam BH, et al. Association between increment of serum VEGF level and prognosis after transcatheter arterial chemoembolization in hepatocellular carcinoma patients. Cancer Sci 2008 Oct;99(10):2037-2044.

(396) Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005 Nov;42(5):1208-1236.

(397) Okuda K, Ohtsuki T, Obata H, Tomimatsu M, Okazaki N, Hasegawa H, et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment. Study of 850 patients. Cancer 1985 Aug 15;56(4):918-928.

(398) A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology 1998 Sep;28(3):751-755.

(399) Zhang ZL, Liu ZS, Sun Q. Expression of angiopoietins, Tie2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma. World J Gastroenterol 2006 Jul 14;12(26):4241-4245.

(400) Deli G, Jin CH, Mu R, Yang S, Liang Y, Chen D, et al. Immunohistochemical assessment of angiogenesis in hepatocellular carcinoma and surrounding cirrhotic liver tissues. World J Gastroenterol 2005 Feb 21;11(7):960-963.

(401) Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003 Jun;9(6):677-684.

(402) Ogasawara S, Yano H, Iemura A, Hisaka T, Kojiro M. Expressions of basic fibroblast growth factor and its receptors and their relationship

to proliferation of human hepatocellular carcinoma cell lines. Hepatology 1996 Jul;24(1):198-205.

(403) Motoo Y, Sawabu N, Yamaguchi Y, Terada T, Nakanuma Y. Sinusoidal capillarization of human hepatocellular carcinoma: possible promotion by fibroblast growth factor. Oncology 1993 Jul;50(4):270-274.

(404) Ribatti D, Vacca A, Roccaro AM, Crivellato E, Presta M. Erythropoietin as an angiogenic factor. Eur J Clin Invest 2003 Oct;33(10):891-896.

(405) Masuda S, Nagao M, Sasaki R. Erythropoietic, neurotrophic, and angiogenic functions of erythropoietin and regulation of erythropoietin production. Int J Hematol 1999 Jul;70(1):1-6.

(406) Kew MC, Fisher JW. Serum erythropoietin concentrations in patients with hepatocellular carcinoma. Cancer 1986 Dec 1;58(11):2485-2488.

(407) Malaguarnera M, Bentivegna P, Di F, I, Laurino A, Romano M, Trovato BA. [Erythropoietin in hepatocellular carcinoma]. Bull Cancer 1996 Dec;83(12):977-980.

(408) Sakisaka S, Watanabe M, Tateishi H, Harada M, Shakado S, Mimura Y, et al. Erythropoietin production in hepatocellular carcinoma cells associated with polycythemia: immunohistochemical evidence. Hepatology 1993 Dec;18(6):1357-1362.

(409) Lowdell MW. Experimental design, data analysis and fluorescence quantitation. In: Macey M, editor. Flow Cytometry: Principles and Applications, 1 ed Humana Press; 2007. p. 133-147.

(410) Khan SS, Solomon MA, McCoy JP, Jr. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin Cytom 2005 Mar;64(1):1-8.

(411) Dulic-Sills A, Blunden MJ, Mawdsley J, Bastin AJ, McAuley D, Griffiths M, et al. New flow cytometric technique for the evaluation of circulating endothelial progenitor cell levels in various disease groups. J Immunol Methods 2006 Oct 20;316(1-2):107-115.

(412) Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000 May 15;95(10):3106-3112.

(413) Arbab AS, Janic B, Knight RA, Anderson SA, Pawelczyk E, Rad AM, et al. Detection of migration of locally implanted AC133+ stem cells by cellular magnetic resonance imaging with histological findings. FASEB J 2008 Sep;22(9):3234-3246.

(414) Furstenberger G, von MR, Lucas R, Thurlimann B, Senn HJ, Hamacher J, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. Br J Cancer 2006 Feb 27;94(4):524-531.

(415) Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 2001 Oct;115(1):186-194.

(416) Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004 Feb;24(2):288-293.

(417) Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003 Mar 4;107(8):1164-1169.

(418) Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG, et al. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res 2003 Nov 28;93(11):1023-1025.

(419) Mellick AS, Plummer PN, Nolan DJ, Gao D, Bambino K, Hahn M, et al. Using the transcription factor inhibitor of DNA binding 1 to selectively target endothelial progenitor cells offers novel strategies to inhibit tumor angiogenesis and growth. Cancer Res 2010 Sep 15;70(18):7273-7282.

(420) Fisher RI, Kaminski MS, Wahl RL, Knox SJ, Zelenetz AD, Vose JM, et al. Tositumomab and iodine-131 tositumomab produces durable complete remissions in a subset of heavily pretreated patients with low-grade and transformed non-Hodgkin's lymphomas. J Clin Oncol 2005 Oct 20;23(30):7565-7573.

(421) Press OW, Unger JM, Braziel RM, Maloney DG, Miller TP, Leblanc M, et al. A phase 2 trial of CHOP chemotherapy followed by tositumomab/iodine I 131 tositumomab for previously untreated

follicular non-Hodgkin lymphoma: Southwest Oncology Group Protocol S9911. Blood 2003 Sep 1;102(5):1606-1612.

(422) Press OW, Eary JF, Gooley T, Gopal AK, Liu S, Rajendran JG, et al. A phase I/II trial of iodine-131-tositumomab (anti-CD20), etoposide, cyclophosphamide, and autologous stem cell transplantation for relapsed B-cell lymphomas. Blood 2000 Nov 1;96(9):2934-2942.

(423) Devizzi L, Guidetti A, Tarella C, Magni M, Matteucci P, Seregni E, et al. High-dose yttrium-90-ibritumomab tiuxetan with tandem stem-cell reinfusion: an outpatient preparative regimen for autologous hematopoietic cell transplantation. J Clin Oncol 2008 Nov 10;26(32):5175-5182.

(424) Salmon HW, Siemann DW. Effect of the second-generation vascular disrupting agent OXi4503 on tumor vascularity. Clin Cancer Res 2006 Jul 1;12(13):4090-4094.

(425) Sugimachi K, Tanaka S, Taguchi K, Aishima S, Shimada M, Tsuneyoshi M. Angiopoietin switching regulates angiogenesis and progression of human hepatocellular carcinoma. J Clin Pathol 2003 Nov;56(11):854-860.

(426) Benetti A, Berenzi A, Gambarotti M, Garrafa E, Gelati M, Dessy E, et al. Transforming growth factor-beta1 and CD105 promote the migration of hepatocellular carcinoma-derived endothelium. Cancer Res 2008 Oct 15;68(20):8626-8634.

(427) De PM, Murdoch C, Venneri MA, Naldini L, Lewis CE. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. Trends Immunol 2007 Dec;28(12):519-524.