MICROBUBBLE ULTRASOUND AS SURROGATE IMAGING BIOMARKER FOR RESPONSE TO SYSTEMIC AND REGIONAL CYTOTOXIC CHEMOTHERAPY

A thesis submitted for the award of Doctor of Philosophy (Ph.D.) Faculty of Medicine Imperial College London

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Professor Paul Abel
Professor Patricia Price
Declaration by candidate

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, these have been acknowledged.

In this study, image acquisition was performed by three trained operators (AM the author of this thesis, with experience of 100 ultrasound liver scans, XF with contrast experience of 10 years, YZ with contrast experience of 5 years).

Image quantification using respiratory gating has been performed by AM.

Signature:

Date: 01-12-2014
Copyright Declaration

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And of course thanks to all the patients for being willing to participate in the clinical research.

Also I am grateful to my funding agency, the Higher Education Commission of Pakistan, for supporting my research at Imperial College London.

My parents for everything, I have!

I dedicate my work to late Dr Tariq Nadeem Ansari, my mentor. You have left unforgettable imprints of kindness, grace, and selflessness on our lives!
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>2-Dimensional</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUWO</td>
<td>Area under the wash out curve</td>
</tr>
<tr>
<td>BD</td>
<td>Bis in die (twice daily)</td>
</tr>
<tr>
<td>BDCEP</td>
<td>Bone marrow derived circulating endothelial cells progenitors</td>
</tr>
<tr>
<td>CECS</td>
<td>Circulating endothelial cells</td>
</tr>
<tr>
<td>CE-HPI</td>
<td>Contrast enhanced hepatic perfusion index</td>
</tr>
<tr>
<td>COV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross sectional area</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DCE</td>
<td>Dynamic contrast enhanced</td>
</tr>
<tr>
<td>DCE-CT</td>
<td>Dynamic contrast enhanced computed tomography</td>
</tr>
<tr>
<td>DCE-MRI</td>
<td>Dynamic contrast enhanced magnetic resonance imaging</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease free survival</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern cooperative oncology group</td>
</tr>
<tr>
<td>DPI</td>
<td>Doppler perfusion index</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxy glucose</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluoro-uracil</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tumours</td>
</tr>
<tr>
<td>HA</td>
<td>Hepatic artery</td>
</tr>
<tr>
<td>HPI</td>
<td>Hepatic perfusion index</td>
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<tr>
<td>ICCC</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>MB</td>
<td>Microbubble</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MBUS</td>
<td>Microbubble ultrasound</td>
</tr>
<tr>
<td>MEV</td>
<td>million electron volts</td>
</tr>
<tr>
<td>MI</td>
<td>Mechanical index</td>
</tr>
<tr>
<td>Mls</td>
<td>millilitres</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>Mean transit time</td>
</tr>
<tr>
<td>MVD</td>
<td>Mean vascular density</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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<tr>
<td>OSR</td>
<td>Overall survival rate</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission tomography</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PI</td>
<td>Peak Intensity</td>
</tr>
<tr>
<td>pO2</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PS</td>
<td>Performance status</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response evaluation criteria for solid tumour</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RT</td>
<td>Rise time</td>
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<tr>
<td>RTHA</td>
<td>Rise time hepatic artery</td>
</tr>
<tr>
<td>RTPV</td>
<td>Rise time portal vein</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PIHA</td>
<td>Peak intensity hepatic artery</td>
</tr>
<tr>
<td>PIPV</td>
<td>Peak intensity portal vein</td>
</tr>
<tr>
<td>SUV</td>
<td>Standardized uptake value</td>
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<tr>
<td>TACE</td>
<td>Transarterialchemoembolisation</td>
</tr>
<tr>
<td>TAE</td>
<td>Transarterial embolisation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>TIC</td>
<td>Time intensity curve</td>
</tr>
<tr>
<td>TIM</td>
<td>Tumour interstitial matrix</td>
</tr>
<tr>
<td>TKIs</td>
<td>Tyrosine kinase inhibitors</td>
</tr>
<tr>
<td>UCA</td>
<td>Ultrasound contrast agents</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WIS</td>
<td>Wash in slope</td>
</tr>
<tr>
<td>WOS</td>
<td>Wash out slope</td>
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</table>
New treatments such as anti-angiogenic therapy, chemoembolisation, radio-embolisation cause tumour stabilisation which may render conventional size based imaging invalid for monitoring early response to therapy. The aim of this thesis was to develop an alternative index which can predict response for patients with liver tumours earlier, at 2 weeks, than the contemporary gold standard response evaluation criteria at 3 months. This technique was based on the use of microbubble ultrasound as an alternative method for detection of hepatic arterialisation.

In this thesis, I evaluated the microbubble ultrasound derived liver blood flow parameters to reflect global liver blood flow in phantom as well as in patients. I also evaluated the impact of several variable factors affecting the liver blood flow and studied the potential role of the blood flow parameters and proposed an alternative index -the contrast enhanced hepatic perfusion index (CE-HPI) - in prediction of early response to treatment for liver tumours. We showed the ability of this technique to reflect global liver blood flow parameters in vitro as well as in patients. Subsequently the index was applied to determine the response assessment in patients receiving treatments.

The results from these experiments present the first demonstration in humans that microbubble ultrasound derived liver blood flow parameters including CE-HPI can be helpful in early identification of non-responders. I propose that the microbubble ultrasound technique developed in this thesis can be used to further probe and investigate response assessment in randomized and multicentre studies.
Relevant Publications

Assessment of global liver blood flow with quantitative dynamic contrast-enhanced ultrasound

T. Gauthier, H. Wasan, A. Muhammad, D. Owen, and E. Leen

Journal of Ultrasound in Medicine, 30 (3), 379-385, March 2011

Reproducibility of quantitative assessment of altered hepatic haemodynamics with dynamic contrast-enhanced ultrasound

T. Gauthier, A. Muhammad, H. Wasan, P. Abel, and E. Leen

Ultrasound in Medicine and Biology, July 2011
TABLE OF CONTENTS

DECLARATION BY CANDIDATE ....................................................................................................................... 2
COPYRIGHT DECLARATION ................................................................................................................................. 3
ACKNOWLEDGEMENTS ........................................................................................................................................ 4
ABBREVIATIONS .................................................................................................................................................. 5
ABSTRACT ............................................................................................................................................................ 8
RELEVANT PUBLICATIONS ............................................................................................................................... 9
TABLE OF FIGURES ........................................................................................................................................... 15
TABLE OF TABLES ............................................................................................................................................ 17
CHAPTER 1: PREMISE AND OBJECTIVES ........................................................................................................ 19
CHAPTER 2 EXPLORATORY FRAMEWORK ..................................................................................................... 22

2.1 CANCER AND IMAGING ............................................................................................................................ 22

2.1.1 RESPONSE ASSESSMENT OF CYTOTOXIC CHEMOTHERAPEUTIC DRUGS AND CYTOSTATIC DRUGS .......................................................................................................................... 22

2.1.2 QUANTIFICATION OF TUMOUR BLOOD FLOW IN FUNCTIONAL IMAGING ............................................. 24

2.1.2.1 DYNAMIC CONTRAST ENHANCED COMPUTED TOMOGRAPHY (DCE-CT) ........................................ 25

2.1.2.2 POSITRON EMISSION TOMOGRAPHY (PET) ...................................................................................... 25

2.1.2.3 DYNAMIC CONTRAST ENHANCED MAGNETIC RESONANCE IMAGING (DCE-MRI) ....................... 26

2.1.2.4 MICROBUBBLE ULTRASOUND ........................................................................................................ 28

2.1.3 SAFETY OF MICROBUBBLES ............................................................................................................... 30

2.1.4 QUANTIFICATION OF BLOOD FLOW WITH MICROBUBBLES ............................................................. 32

2.1.5 PHYSICS OF MICROBUBBLE NON-LINEARITY AND CONTRAST SPECIFIC IMAGING TECHNIQUES ............................................................................................................................. 33

2.1.6 APPROACHES FOR QUANTIFICATION OF TUMOUR BLOOD FLOW ...................................................... 38

2.1.7 TECHNIQUES FOR COMPENSATION OF RESPIRATORY MOTION .................................................... 40

2.2 IMAGING AS A BIOMARKER IN CANCER TREATMENT ............................................................................. 42

2.3 ANGIOGENESIS AND ANTIANGIOGENIC THERAPY ................................................................................ 43

2.3.1 HOW DO TUMOUR BLOOD VESSELS DIFFER FROM NORMAL BLOOD VESSELS? ............................... 46

2.3.2 ANTIANGIOGENIC THERAPY .................................................................................................................. 48
2.3.3 CYTOTOXIC CHEMOTHERAPY AND ANTIANGIOGENIC EFFECTS ........................................50
2.3.4 SELECTIVE INTERNAL RADIATION THERAPY (SIRT) ..................................................51
2.3.5 ASSESSMENT OF VASCULARITY AND ANGIOGENESIS, SIGNIFICANCE AND CHALLENGES ..52
2.4 LIVER HAEMODYNAMICS ..........................................................................................53
  2.4.1 POST PRANDIAL VARIABILITY AND LIVER HOMEOSTASIS ........................................54
  2.4.2 MEASUREMENT OF LIVER HAEMODYNAMICS ...........................................................56
  2.4.3 DOPPLER PERFUSION INDEX (DPI) .............................................................................57
  2.4.4 CONTRAST ENHANCED DOPPLER PERFUSION INDEX (CE-DPI) ...............................59
  2.4.5 TUMOUR PERFUSION STUDIES ................................................................................60
  2.4.6 CONTRAST ENHANCED HEPATIC PERFUSION INDEX (CE-HPI) .................................64

CHAPTER 3 METHODS ........................................................................................................66
  3.1 METHOD OF RECRUITMENT, SAMPLE SIZE AND GAINING PATIENTS' CONSENT ............67
  3.2 INTERVENTIONS AND TREATMENT REGIMENS ............................................................68
  3.3 DCE-US SCHEDULE ......................................................................................................68
  3.4 IMAGING PROTOCOL .................................................................................................69
    3.4.1 OPERATORS ............................................................................................................69
    3.4.2 IMAGING EQUIPMENT ............................................................................................69
    3.4.2.1 CONTRAST AGENT ............................................................................................69
    3.4.2.2 METHOD OF PREPARATION OF SONOVUE: VIAL PREPARATION .......................70
    3.4.2.3 METHOD OF INJECTION ....................................................................................72
    3.4.2.4 PHARMACOKINETICS AND PHARMACODYNAMICS OF MICROBUBBLES ..........73
    3.4.2.5 ULTRASOUND SCANNER .................................................................................73
    3.4.2.6 ULTRASOUND SCANNER SETTINGS ..................................................................73
    3.4.3 IMAGING PROTOCOL FOR THE EVALUATION OF LIVER BLOOD FLOW PARAMETERS......75
    3.4.4 SAFETY MONITORING ............................................................................................77
    3.4.5 IMAGE TRANSFER ....................................................................................................77
    3.4.6 QLAB COMMERCIAL QUANTIFICATION SOFTWARE .............................................78
  3.5 IMAGE ANALYSIS: ......................................................................................................78
4.6 TITLE: ASSESSMENT OF ALTERED HEPATIC BLOOD FLOW PARAMETERS IN PATIENTS WITH LIVER TUMOURS WITH SELECTIVE INTERVAL RADIOThERAPY TREATMENT (SIRT) .......................... 143

BACKGROUND .................................................................................................................. 143

PATIENT RECRUITMENT .................................................................................................. 144

METHODS .......................................................................................................................... 145

PROTOCOL .......................................................................................................................... 145

RESULTS .............................................................................................................................. 146

DISCUSSION ....................................................................................................................... 149

SUMMARY ........................................................................................................................... 141

CHAPTER 5: FINAL DISCUSSION AND CONCLUSION OF THESIS ...................................... 152

COMPARISON WITH PAST STUDIES .................................................................................. 157

CHALLENGES, LIMITATIONS AND POTENTIAL SOLUTIONS ........................................... 159

PRACTICAL APPLICATIONS OF THIS WORK .................................................................... 164

SUMMARY AND CONCLUSION ......................................................................................... 167

FUTURE WORK .................................................................................................................... 167

REFERENCES ...................................................................................................................... 168

APPENDIX 1: PERFORMANCE STATUS (PS) ....................................................................... 178

APPENDIX 2: IMPORTANT FUNCTIONS ON QLAB SOFTWARE ........................................ 179

APPENDIX 3: QLAB PROTOCOL ......................................................................................... 185

APPENDIX 4: COMPARISON OF IMAGING TECHNIQUES FOR QUANTIFICATION OF BLOOD FLOW 189

APPENDIX 5: CYTOTOXIC CHEMOTHERAPEUTIC REGIMENS ......................................... 190

APPENDIX 6: SUMMARY OF TUMOUR PERFUSION STUDIES ............................................ 196
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>DCE-MRI for response assessment and evaluation of antiangiogenic activity</td>
<td>23</td>
</tr>
<tr>
<td>Figure 2</td>
<td>DCE-MRI for response assessment and evaluation of antiangiogenic activity</td>
<td>27</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Ultrasound signal intensity</td>
<td>33</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Frequency spectrum of an echo produced by microbubbles</td>
<td>34</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Pulse inversion harmonic imaging</td>
<td>35</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Power modulation harmonic imaging</td>
<td>36</td>
</tr>
<tr>
<td>Figure 7</td>
<td>The contrast bolus injection technique</td>
<td>39</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Blood volumetric flow</td>
<td>45</td>
</tr>
<tr>
<td>Figure 9A</td>
<td>Vascular system of normal tissue</td>
<td>46</td>
</tr>
<tr>
<td>Figure 9B</td>
<td>Vascular system of a solid tumour</td>
<td>47</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Normalisation window</td>
<td>49</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Contrast phases in the liver, as indicated by time intensity curves</td>
<td>65</td>
</tr>
<tr>
<td>Figure 12</td>
<td>SonoVue, consisting of unilayer of phospholipid shell,</td>
<td>69</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Package of the commercial microbubble based agent SonoVue (Bracco, Milan, Italy)</td>
<td>70</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Steps to prepare the SonoVue (microbubbles) vial prior to injection</td>
<td>71</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Method of SonoVue microbubble injection</td>
<td>72</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Step 1: Identification of bright reflector (generally diaphragm) on brightness mode</td>
<td>79</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Step 2: Selection of diaphragm and region of interest:</td>
<td>79</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Step 3: Drawing region of interest</td>
<td>80</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Step 4: Time intensity curve for diaphragm rhythmic movements</td>
<td>80</td>
</tr>
<tr>
<td>Figure 20A</td>
<td>Curves without respiratory gating</td>
<td>81</td>
</tr>
<tr>
<td>Figure 20B</td>
<td>Step 6: Comparatively smooth curves after respiratory gating</td>
<td>82</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Example of the smoothened time intensity curves derived from hepatic artery and portal vein, after respiratory gating</td>
<td>83</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Indicator dilution setup</td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 23. A, B, C ratios of the RT, PI, and WIS in the 2 mm tube to those measured in the 5 mm tube as a function of ratio of volumetric flow in the 2 mm tube to 5 mm tube.

Figure 24. Bland-Altman chart for test re-test reliability of CE-HPI.

Figure 25. Bland-Altman chart for inter-operators variability of CE-HPI.

Figure 26. Bland-Altman chart for variability of CE-HPI in fasting vs. non-fasting state.

Figure 27. A, B, C, D. Bland-Altman plots for PI ratio inter-reader and inter-scan and CE-HPI inter-reader and inter-scan respectively.

Figure 28. Patient recruitment summary.

Figure 29. PI ratio: comparison between responders and non-responders.

Figure 30. CE-HPI: comparison between responders and non-responders.

Figure 31. Patient recruitment summary.

Figure 32. PI ratio at 2 weeks: comparison between responders and non-responders.

Figure 33. CE-HPI at 2 weeks: comparison between responders and non-responders.

Figure 34. Patient recruitment summary.

Figure 35. Peak intensity ratio: comparison between responders and non-responders.

Figure 36. CE-HPI: comparison between responders and non-responders.

Figure 37. Patient recruitment summary for SIRT.

Figure 38. PI ratio for comparisons of effects of SIRT at different time intervals.

Figure 39. CE-HPI for comparisons of effects of SIRT at different time intervals.
Table of tables

TABLE 1. MICROBUBBLE CONTRAST AGENTS FOR ULTRASOUND .................................................. 29
TABLE 2. TYPES OF MICROBUBBLES AND THEIR COMPOSITION.............................................. 29
TABLE 3. REVIEW OF STUDIES CONDUCTED FOR SAFETY ANALYSIS OF MICROBUBBLES .................. 31
TABLE 4. BIOLOGICAL AGENTS AND MOLECULAR TARGETS....................................................... 48
TABLE 5. SUMMARY OF MACHINE SETTINGS FOR CONTRAST SPECIFIC IMAGING IN THIS THESIS .................. 75
TABLE 6. PATIENT CHARACTERISTICS AND DEMOGRAPHICS AT BASELINE .................................. 95
TABLE 7. PEAK INTENSITY RATIO OF PATIENTS AND HEALTHY CONTROLS ..................................... 96
TABLE 8. CE-HPI OF PATIENTS AND HEALTHY CONTROLS ............................................................. 96
TABLE 9. WIS RATIO AND RISE TIME RATIO OF PATIENTS AND HEALTHY CONTROLS ..................... 96
TABLE 10. TEST RE-TESTS (INTER-SCAN) RELIABILITY OF CE-HPI .................................................. 101
TABLE 11. INTER-OPERATOR VARIABILITY OF CE-HPI .................................................................... 102
TABLE 12. CE-HPI VARIABILITY IN FASTING VS. NON-FASTING STATE ........................................... 104
TABLE 13. BLOOD FLOW PARAMETER VARIABILITY IN FASTING VS. NON-FASTING STATE ..................... 105
TABLE 14. SUMMARY OF REPRODUCIBILITY STUDIES BY DIFFERENT RESEARCH GROUPS .................. 107
TABLE 15. PI RATIO, AND WIS RATIO VALUES MEAN (SD) FOR PATIENTS WITH COLORECTAL LIVER METASTASES ........................................................................ 114
TABLE 16. COEFFICIENTS OF VARIATION (SD/Mean) FOR INTER-READER, INTER SCAN VARIABILITY ......................................................... 114
TABLE 17. INTRACLASS CORRELATION COEFFICIENTS FOR INTER-READER AND INTER-SCAN VARIABILITY ........................................................................ 114
TABLE 18. SUMMARY OF PATIENT CHARACTERISTICS ..................................................................... 120
TABLE 19. PI RATIO: COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS ..................... 121
TABLE 20. CE-HPI COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS .......................... 122
TABLE 21. PFS AND OS FOR RESPONDERS AND NON-RESPONDERS ............................................... 123
TABLE 22. SUMMARY OF PATIENT CHARACTERISTICS AND DEMOGRAPHICS .............................. 125
TABLE 23. PI RATIO AT 2 WEEKS: COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS .................. 125
TABLE 24. CE-JPI AT 2 WEEKS COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS ............... 126
TABLE 25. PFS AND OS FOR RESPONDERS AND NON-RESPONDERS .............................................. 128
TABLE 26. SUMMARY OF PATIENT CHARACTERISTICS ..................................................................... 136
TABLE 27. PI RATIO: COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS .......................................................... 136

TABLE 28. CE-HPI COMPARISON BETWEEN RESPONDERS AND NON-RESPONDERS .......................................................... 137

TABLE 29. PFS AND OS FOR RESPONDERS AND NON-RESPONDERS .......................................................... 139

TABLE 30. PATIENTS’ DEMOGRAPHICS AT BASELINE ................................................................................................. 145

TABLE 31. PI RATIO FOR COMPARISONS OF EFFECTS OF SIRT AT DIFFERENT TIME INTERVALS .................................... 147

TABLE 32. CE-HPI FOR COMPARISONS OF EFFECTS OF SIRT AT DIFFERENT TIME INTERVALS .................................... 148

TABLE 33. SUMMARY OF THE THESIS FINDINGS ........................................................................................................ 155

TABLE 34. SUMMARY OF THE THESIS FINDINGS—PROGRESSION FREE SURVIVAL AND OVERALL SURVIVAL ............ 155
Chapter 1: Premise and Objectives

Liver is one of the body’s unique organs, with a dual blood supply, and the second largest (after skin). Due to its large size and extensive blood supply, the liver is one of the most common sites for metastases. When these develop, significant changes occur due to the associated arterialisation. Normally the liver receives about 33% of its blood supply through the hepatic artery, with the remainder being provided via the portal vein [1]. Studies have shown that metastatic liver tumours may receive up to 90% of their blood supply from the hepatic artery [2]. In other words, the normal proportions of blood supplied by these two vessels are reversed.

Various researchers have described the application of the Doppler ultrasound technique in non-invasive measurement of increased arterialisation of the liver blood supply following tumour formation [3-5]. It has been suggested that an elevated doppler perfusion index (DPI), which is defined as the ratio of the hepatic arterial volume flow to the sum of the hepatic artery and portal vein volume flow, is a useful diagnostic indication of the presence of liver metastases [6]. Leen et al., in 2000, were the first to show that DPI can be used to correctly identify colorectal cancer (CRC) patients with high risk of recurrence, to enable them to receive appropriate cytotoxic chemotherapy, post-operatively. In their study they found that, patients with high DPI > 0.30 had 5-years disease free survival (DFS) of 22% and 5-years overall survival (OS) of 29% compared to patients with normal DPI, who had rates of 89% DFS, and 91% OS [7].

Unfortunately, DPI measurements are technically difficult and time consuming to perform (due to issues with beam angles and inaccurate measurement of the areas of the vessel studies) and are strongly operator dependent. With the advent of microbubbles (ultrasound
contrast agents), microbubble ultrasound has been proposed as an alternative technique for the detection of hepatic arterialisation, particularly in patients with hepatic cirrhosis \[8, 9\]. Early reports with small patient numbers and varying primary tumours suggest that this technique can also be used to demonstrate increased arterialisation developed through liver metastases \[10\].

In this thesis, we have proposed and developed a method based on microbubble ultrasound to provide an alternative index—contrast-enhanced hepatic perfusion index (CE-HPI)—for the measurement of blood flow changes in the liver, which is defined as the ratio of the volumetric blood flow in the hepatic artery to the volumetric blood flow in the portal vein.

This index may be particularly more useful in monitoring the response to new biological agents such as bevacizumab, cetuximab and sunitinib for treating liver tumours. These biological agents are small biological molecules which mainly target tumour microcirculation (antiangiogenic effect) and consequently are cytostatic, causing either tumour stabilisation or increase in size due to oedema or necrosis \[11\]. In addition to biological agents, cytotoxic chemotherapy regimens (such as weekly taxanes in breast cancer) have also been suggested to have an antiangiogenic effect when given more frequently in low doses\[12\].

Conventional imaging techniques based on response revaluation criteria in solid tumours (RECIST) may be of limited use in the early assessment of tumour response to these agents, as they rely on the measurement of changes in tumour size, which is a late event. Functional modalities such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), dynamic contrast enhanced computed tomography (DCE-CT) and dynamic contrast
enhanced ultrasound (DCE-US) may be better options for assessing response to these agents. DCE-US has the further advantage that microbubbles do not extravasate and are true intravascular blood pool contrast agents[13]. Also the backscattered ultrasound intensity is linearly proportional to microbubbles in low to moderate concentration, which means different liver blood flow parameters in these patients can be quantified using time intensity curves (TIC) before and after treatment [14]. I hypothesise that measurement of CE-HPI predicts an early response to cytotoxic chemotherapy, biological treatments and radio-embolisation, and is an alternative imaging biomarker for early response to these treatments. The aim of this research is to test this hypothesis as follows:

1. To establish sonographic parameters of liver blood flow in phantom and in a group of patients with liver tumours who are undergoing active treatment that targets the tumour microvasculature and

2. To validate microbubble ultrasound as an early predictor of response to cancer treatment with cytotoxic chemotherapy, novel biological targeted agents and radio-embolisation

If this hypothesis is confirmed, a non-invasive imaging biomarker will enable clinicians to gauge the probable efficacy of treatment. This would give them the option of switching to a different therapy or adjusting the dosage schedule. Patients would be able to avoid unnecessary treatment from which they may not derive benefit and which may have significant side-effects, thus reducing costs and improving outcomes.
Chapter 2 Exploratory framework

2.1 Cancer and imaging

The medical use of ultrasound (US) began in various centres around the world after the 2nd World War. In the mid-1950s, Professor Ian Donald, a Scottish physician, facilitated the development of practical technology and applications in US[15]. In conjunction with conventional US imaging, the development of Doppler US increased the range for investigation of the blood supply of tumours and different body organs[16]. Later, in the 1970s, Godfrey Hounsfield invented computed tomography (CT), which showed detailed images of various structures inside the body, including blood vessels, bones, internal organs, and tumours, using computers and serial x-rays [17].

During the same period scientists demonstrated that different body tissues resonate at different magnetic field strengths, and images of the scanned areas of the body can be extracted from these magnetic fields. This technique was called magnetic resonance imaging (MRI) [18]. The development of CT scanning and MRI facilitated the response assessment of various treatments, including cytotoxic chemotherapy in cancer patients.

2.1.1 Response assessment of cytotoxic chemotherapeutic drugs and cytostatic drugs

As MRI and CT scans became widely available for tumour size measurement, by the late 1990s an international working group had started collaborating to establish the criteria for response assessment in solid tumours [19]. Depending on the response, the following four categories were defined [20]: progressive disease (PD) is ≥ 20% increase in the sum of diameter of lesions, whilst appearance of any new disease also constitutes PD. Partial
response (PR) is ≥ 30% reduction in the sum of diameter of lesions, as compared to baseline assessment. Meanwhile, stable disease (SD) is neither sufficient increase in size of the lesions to constitute PD, nor sufficient decrease to constitute PR, whilst complete response (CR) indicates disappearance of all target lesions. These conventional RECIST based techniques have certain limitations. New treatment modalities such as cytostatic agents (targeted therapies such as sorafenib, cetuximab and bevacizumab), and few cytotoxic chemotherapeutic regimens such as weekly docetaxel in breast cancer, mainly arrest cell division and growth initially, which may render conventional imaging criteria like RECIST invalid for monitoring response to these agents (figure 1).

![A. Baseline CT scan](image1.png)  ![B. Post-treatment scan (6 weeks of Sunitinib)](image2.png)

**Figure 1.** DCE-MRI for response assessment and evaluation of antiangiogenic activity in a patient with renal cell cancer and liver metastases. AUC90 (area under the concentration curve 90 seconds after the contrast injection) which indicates liver tumour blood volume shows reduction in 4 weeks after treatment with sorafenib. Adapted from [21].
Alternative functional imaging modalities, such as dynamic contrast enhanced CT (DCE-CT) and DCE-MRI, are contrast enhanced monitoring techniques that rather than simple assessment of physical size changes, rely on intracellular physiological processes in tumours, such as change in tumour density and perfusion, permeability, blood volume, and tumour blood flow (microcirculation) [22, 23]. These techniques entail capturing features of the temporal and spatial distribution of the contrast agents as a time intensity curve (TIC) from a series of contrast enhanced images, which could be helpful in quantification of tumour blood flow.

**2.1.2 Quantification of tumour blood flow in functional imaging**

Imaging and quantification of tumour blood flow (flow rate, velocity, volume and morphology) has been a significant challenge. In a typical single capillary, flow velocity is about 0.3 mm/s, vessel diameter is about 5 µm, and flow rate less than 1 ml per year[24]. With the help of contrast agents, the functional and physiological parameters of microcirculation can be determined by monitoring the kinetics and tissue distribution of an intravenously injected tracer material or contrast agent.

Contrast agents can be classified into two distinct groups: Intravascular tracers, such as microbubble ultrasound contrast agents (UCAs), are confined to the blood pool and possess similar kinetics to that of red blood cells[25]. Diffusible contrast agents are much smaller in size than their intravascular counterparts, and can leak through vessel walls into the surrounding extra vascular space. The diffusible agents used in DCE-CT scan, PET and DCE-MRI include Gadolinium and contrast iodine [26].
2.1.2.1 Dynamic contrast enhanced computed tomography (DCE-CT)

In DCE-CT, after the administration of a contrast agent in the form of IV bolus, different time intensity curves are acquired with consecutive scans and then analysed. Pharmacokinetic modeling of CT contrast agents takes into account diffusion of tracer particles out of the blood pool, and thus shares similarities with DCE-MRI [27].

DCE-CT can help in determination of relative blood volume, capillary permeability, and tissue perfusion, and thus can act as an in vivo cancer angiogenesis biomarker. Recently it has been used more frequently in clinical and research settings for evaluation of antiangiogenic and antivascular biological agents [28]. Furthermore, dynamic or functional CT can be easily combined with routine conventional CT in a technique that is reproducible, simple and widely available [27]. The relationship between contrast concentration and signal is linear in comparison to MRI, which makes quantification of CT simpler than that of MRI [29].

Nevertheless, DCE-CT has a few limitations. Multiple imaging assessments are required of tumours for early clinical studies of antiangiogenic compounds, whereas the number of studies that can be performed in each patient is limited, due to the ionizing radiation in DCE-CT [29].

2.1.2.2 Positron Emission Tomography (PET)

PET imaging has a wide variety of tracers that are specific to each type of measurement (\(^{15}\)O-water for perfusion, \(^{15}\)O-carbon monoxide for vascular volume, \(^{18}\)fluoro fluorodeoxy glucose positron emission tomography (\(^{18}\)F-FDG) for glucose metabolism etc.) [30]. Various
oncologic studies have provided evidence which supports the use of $^{18}$F-FDG PET not only in treatment planning for different tumours, but also for response assessment [31]. The standard uptake value (standardized measure of radioactivity uptake in the region of interest) has been found to have a positive role in response assessment in several tumours, whilst $^{18}$F-FDG PET has also been shown to enhance the reliability of patient survival prediction [31].

PET as an imaging biomarker has the advantage as compared to biomarkers such as tumour biopsies and plasma samples of allowing non-invasive and serial assessment of the whole tumour; whereas, biopsies are derived from a small portion of the tumour and heterogeneity within the tumour may be missed. However, as the spatial resolution of PET imaging is in the range of 5 to 6mm, it therefore has limited use in the evaluation of small lesions and tumour heterogeneity [32]. Moreover, response assessment in antiangiogenic therapy requires multiple and serial scans, which restricts the routine use of PET due to radiation hazards.

2.1.2.3 Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI)

In DCE-MRI, after a low molecular weight paramagnetic contrast agent (e.g. Gadolinium) has been injected in intravenous bolus form, its passage is tracked through the tumour vasculature, following which any changes in intensity of the repeatedly acquired T1-weighted DCE-MR Image can be converted into data of contrast agent concentration. Further kinetic modeling is applied in order to produce modeled parameters which are sensitive to physiologic processes like tissue micro-vessel perfusion, permeability, and extracellular extra vascular leakage space. Antiangiogenic effects of cancer treatments can
then be evaluated by changes in these parameters [33]. During treatment, alterations in vascularity of the tumour occur earlier than tumour size changes and early clinical trials with antiangiogenic and vascular-disrupting compounds are increasingly employing DCE-MRI as a surrogate biomarker as it provides good spatial resolution, without involvement of ionizing radiation [34]. Practical applications of DCE-MRI include monitoring tumour response by measurement and comparison of relative change in signal enhancement from baseline to post-treatment (figure 2) [26].

Figure 2. DCE-MRI for response assessment and evaluation of antiangiogenic activity in a patient with renal cell cancer and liver metastases. IAUC90 (area under the concentration curve 90 seconds after...
the contrast injection indicates liver tumour blood volume) shows reduction within 4 weeks after treatment with sorafenib. Adapted from [21].

Despite showing promising indications, DCE-MRI has many limitations; in particular, absolute quantification with DCE-MRI is difficult, because the relation between Gadolinium concentration and MR signal intensity is complicated [35]. Another limitation of DCE-MRI is the contrast agents, which are extra vascular and easily pass into the interstitial space. Ultrasound contrast agents (UCA), on the other hand, are truly intravascular and do not extravasate [13]. Due to the intravascular nature of UCA, tissue perfusion is accurately determined, as microbubbles contrast agent perfusion is equivalent to the distribution of blood [36].

2.1.2.4 Microbubble ultrasound

Microbubbles were first discovered in 1968 by Gramiak and Shah, when they injected agitated saline into the aorta, and were able to opacify the walls and delineate the border of the right ventricle [37]. The agitated saline was prepared by quickly injecting normal saline from one vial into another through a 3-way tube, so that air became trapped in it, and mixing it with the patient’s blood. The microbubbles were unstable and active for a very short duration of less than 10 seconds [37].

Discovery of this phenomenon by Gramiak and Shah generated vast interest in the field of contrast ultrasound, which led to development of first generation air filled microbubbles. However, these first generation microbubbles, such as Echovist (consisting of air bubbles), lacked stabilisation shells and could not pass through the capillaries due to their large size (table 1) [38].
The Evolution Of Contrast Agents For Ultrasound

<table>
<thead>
<tr>
<th>Generation</th>
<th>Formulation</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Free gas bubbles</td>
<td>Cannot pass through cardiopulmonary beds</td>
</tr>
<tr>
<td>1</td>
<td>Encapsulated air bubbles</td>
<td>Successful transpulmonary passage</td>
</tr>
<tr>
<td>2</td>
<td>Low solubility gas bubbles: encapsulated</td>
<td>Improved stability</td>
</tr>
<tr>
<td>3</td>
<td>Polymer shell bubbles</td>
<td>Controlled acoustic properties</td>
</tr>
</tbody>
</table>

Table 1. Microbubble contrast agents for ultrasound. Adapted from [39]

The low solubility of first generation air filled microbubbles in the peripheral circulation was overcome by formation of more stable bubbles with galactose, palmitic acid or phospholipid shells. These 2nd generation microbubbles, such as Levovist and Albunex, were of almost the same size as red blood cells (RBCs), and hence could easily pass through pulmonary capillaries. Due to the high molecular weight and low solubility of gases (such as air) stabilised by microbubble shells, these microbubbles persist for several minutes in peripheral circulation, giving sufficient time for ultrasound examination.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Manufacturer</th>
<th>Coating</th>
<th>Gas</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue</td>
<td>Bracco</td>
<td>Phospholipid</td>
<td>SF6(sulphur hexafluoride)</td>
</tr>
<tr>
<td>Definity</td>
<td>Lantheaus medical imaging</td>
<td>Phospholipid</td>
<td>octafluoropropane</td>
</tr>
<tr>
<td>Optison</td>
<td>GE healthcare</td>
<td>Protein (human serum albumin)</td>
<td>octafluoropropane</td>
</tr>
<tr>
<td>Sonazoid</td>
<td>GE healthcare</td>
<td>lipids</td>
<td>Perfluoro-butane</td>
</tr>
<tr>
<td>Levovist</td>
<td>Schering</td>
<td>Lipid</td>
<td>Air</td>
</tr>
<tr>
<td>Albunex</td>
<td>Mallinckrodt</td>
<td>Protein (human serum albumin)</td>
<td>Air</td>
</tr>
</tbody>
</table>

Table 2. Types of microbubbles and their composition. Adapted from [40].

Third generation microbubbles work at a low mechanical index (explained on page 36) in sophisticated harmonic imaging systems. They have gases which are only sparingly soluble
in blood and are physiological inert, so their half-life is improved to 15 minutes; examples include SonoVue, Definity, and Luminity (table 2). This new generation of microbubbles has also been designed with a view to maintaining their safety.

2.1.3 Safety of microbubbles

Potential safety of contrast agents such as SonoVue has been extensively investigated in animals (rats and monkeys) and humans. High doses of > 20 ml/kg body weight have not shown any toxic side effects [41]; incidence of anaphylactic reaction is <0.001 % (1 in 1000), headache (2.3 %), injection site pain (1.4%), injection site bruising, burning, and paraesthesia (1.7 %) (Bracco SonoVue SPC, 2005). These agents are non-nephrotoxic and neither do they affect the thyroid [42].

In October 2007, the Food and Drug Administration (FDA) issued a black box warning (the strongest type of warning appearing on the package insert for prescription drugs) on receiving reports of 4 deaths and 190 serious cardiopulmonary reactions. Although there was a close temporal relationship, no clear causal relationship to contrast injection was established. Besides issuing a black box warning, the FDA also issued multiple disease state contraindications and a mandatory 30 minute monitoring period for patients undergoing contrast ultrasound examination.

Piscaglia et al. conducted a retrospective analysis on a total of 23188 investigations using SonoVue, 99% of which were performed on the liver [43]. A total of 28 Italian centres participated in a large European study. A whole vial (4.8 ml) dose was generally used, whilst 50 % to 60 % of the cases were given 1.2 ml to 2.4 ml. No fatal events or serious side effects (death, hospitalisation, disability, life threatening, congenital anomaly, and birth defect)
were recorded, whilst only two serious events were reported, which included dyspnoea, bronchospasm, hypotension, rash, with 23 non-serious events reported. Of these only 4 events required treatment. Overall the serious adverse rate was 0.0086 %, which is less than with any other contrast agent, while the non-serious side effect profile was temporary and did not lead to disability. This large scale study showed that SonoVue has a good safety profile in abdominal applications[43].

Following the publication of this large European study, the FDA removed the warning for multiple disease state contraindications, and the 30 minutes mandatory monitoring period, but retained the black box warning for high risk patients with severe pulmonary hypertension and unstable cardiopulmonary conditions [44].

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Contrast examinations</th>
<th>Non-contrast examinations</th>
<th>Primary outcome</th>
<th>Odds ratio for mortality (1 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main et al.[45]</td>
<td>2008</td>
<td>58254</td>
<td>4242712</td>
<td>Mortality (1 day)</td>
<td>0.76</td>
</tr>
<tr>
<td>Kusnetzky et al.[46]</td>
<td>2008</td>
<td>6196</td>
<td>12475</td>
<td>Mortality (1 year)</td>
<td>1.14</td>
</tr>
<tr>
<td>Herzog et al.[47]</td>
<td>2008</td>
<td>96751</td>
<td>16025</td>
<td>Adverse events</td>
<td>No deaths</td>
</tr>
<tr>
<td>Wei et al.[48]</td>
<td>2008</td>
<td>780243</td>
<td>78383</td>
<td>Adverse events</td>
<td>No deaths</td>
</tr>
<tr>
<td>Gabriel et al.[49]</td>
<td>2008</td>
<td>4786</td>
<td>5012</td>
<td>Adverse events and mortality (1 day)</td>
<td>No deaths</td>
</tr>
<tr>
<td>Shaikh et al.[50]</td>
<td>2008</td>
<td>2914</td>
<td>2155</td>
<td>Adverse events and mortality</td>
<td>No deaths</td>
</tr>
<tr>
<td>Abdelmoneim et al. [51]</td>
<td>2009</td>
<td>10792</td>
<td>15982</td>
<td>Adverse events and mortality (72 hours)</td>
<td>0.99</td>
</tr>
<tr>
<td>Dolan et al.[52]</td>
<td>2009</td>
<td>42408</td>
<td>5900</td>
<td>Adverse events and mortality (72 hours)</td>
<td>0.25</td>
</tr>
<tr>
<td>Goldberg et al.[53]</td>
<td>2012</td>
<td>2518</td>
<td>94187</td>
<td>Mortality (1 day)</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Table 3. Review of studies conducted for safety analysis of microbubbles
To further address the concerns of the FDA, Goldberg et al. (2012) conducted a study which reviewed 96705 ultrasound examinations over a period from July 2003 to June 2008. They found 0.44 % day1 mortality in the contrast scan group, and 0.45 % mortality in the control group. Meanwhile, multivariate analysis did not show any increase in mortality after adjustment for factors which included age, gender, inpatient versus outpatient and left ventricular ejection fraction (adjusted odds ratio 1.18 %, 95 % confidence interval 0.56 to 2.48, p=0.67[54]. Another large meta-analysis (table 3) of ultrasound contrast agent safety studies consisted of 211,162 contrast administrations, and 5078666 non-contrast echocardiograms acting as controls. The contrast echocardiography pooled odds ratio for mortality was 0.57 (95 % confidence interval 0.32-1.01), results which reassured both clinicians and regulators. Such safety reassurance encouraged the current researcher to expand the work on quantification of blood flow with microbubbles.

2.1.4 Quantification of blood flow with microbubbles

Quantification of contrast ultrasound is based on the principle that ultrasound signal intensity is proportional to the concentration of microbubbles in any area of interest, such as tumours, normal tissue, hepatic artery or portal vein (figure 3)[14]. As microbubble ultrasound is a real time imaging technique with high spatial and temporal resolution, it is sensitive for measurement of very slow blood flow in micro vessels (<1mm/second). Nevertheless, microbubble ultrasound has certain limitations. Capillary vascular permeability cannot be assessed with microbubbles due to their larger size, which also prevents them from crossing the endothelial membranes [55].
At high acoustic power microbubble rupture can occur, limiting the depth of tissue which can be visualized clearly on the ultrasound [56]. Consequently contrast specific imaging techniques have been developed which are based on the physics of microbubbles induced non-linear echoes.

2.1.5 Physics of microbubble non-linearity and contrast specific imaging techniques

An ultrasound wave consists of compressions (areas of high pressure) and rarefactions (areas of negative pressure). During compression, microbubbles are squeezed, leading to reduction in diameter, whilst during rarefactions, they are dilated. Both dilations and expansions of microbubbles are several hundred percent of the original size, whereas any decrease in size during compressions is limited due to the gas having low compressibility. As
a result, when exposed to ultrasound waves, microbubbles oscillate in a non-linear behaviour, which leads to a frequency spectrum containing fundamental frequencies similar to incidental ultrasound waves and harmonic components (i.e. multiples of fundamental frequency of 2 x, 3 x, 4x or even more).

Figure 4. Frequency spectrum of an echo produced by microbubbles. On exposure to an ultrasound wave with 3-MHz frequency, in addition to the fundamental echo at 3 MHz, harmonics which are multiples of fundamental / transmitted frequency are also generated. Adapted from [57].

Harmonic components are essential to harmonic imaging.

**Harmonic imaging**

Harmonic imaging is the mode in which the transducer is adjusted to receive harmonics.

Harmonics are multiples of fundamental frequency which form a non-linear effect within the tissue. There are three main types of contrast imaging techniques:
Low MI pulse inversion /phase inversion technique

In the pulse inversion technique, two pulses are sent consecutively and the second pulse is inverted (180° out of the phase). In the linear mode, consecutive signals cancel out each other and the sum is zero, while in the non-linear mode, due to asymmetric contractions, signals do not cancel each other out and instead result in image enhancement of the blood flow.

![Diagram of pulse inversion harmonic imaging](image)

Figure 5. Pulse inversion harmonic imaging. Pulse inversion technique cancels out fundamental echoes while preserving harmonic echoes. Due to the differing action of the gas in relation to compression and expansion, microbubbles respond differently to positive and negative pressure, resulting in an asymmetrical response, and hence the received signals do not summate to zero. Adapted from [56].

Low MI amplitude modulation /power modulation

In the amplitude /power modulation technique, instead of the angle of the phase being changed by 180°, the amplitude of the pulses is changed. For example, if 3 pulses are sent in succession, the first will be at half amplitude, the second at full amplitude, while the third will again be at half amplitude. Low amplitude pulses generate fewer harmonics than those of high amplitude. The machine is calibrated to pick up only high amplitude harmonics and
to subtract low amplitude signals. These high amplitude pulses enhance only non-linear signals from the microbubbles.

**Figure 6. Power modulation harmonic imaging.** The nonlinear components are grouped at the fundamental and at higher harmonics. Adapted from [56].

**Low MI pulse inversion / amplitude modulation**

The third technique is a combination of pulse inversion and power modulation imaging techniques.

**Mechanical Index**

The mechanical index is a safety indicator of ultrasound for biological effects such as cavitation and is calculated as a ratio of peak negative acoustic pressure to square root of centre frequency.

\[
\text{Mechanical index} = \frac{\text{Peak negative pressure}}{\sqrt{\text{frequency}}}
\]

Peak negative pressure is also a safety indicator and a measure of the inertial cavitation and harmful effects. Cavitation, meanwhile, is a phenomenon due to negative pressure, and
indicates a response of gas bubble formation in a liquid medium under the influence of sound waves.

At high MI > 1.2 microbubbles collapse and rupture, resulting in emission of strong non-linear echoes that is called simulated acoustic emission imaging.

At moderate MI 0.06-1.2 asymmetric oscillations are produced, resulting in non-linear behaviour due to expansion being greater than contraction.

At low ultrasound MI <0.06 linear, symmetrical response occurs due to equal expansion and contraction.

Due to these symmetric responses, determination of microbubble ultrasound derived blood flow parameters is possible for quantification of tumour blood flow, without destruction of the microbubbles.
2.1.6 Approaches for quantification of tumour blood flow

There are two different microbubble ultrasound approaches for quantification of tumour blood flow: bolus technique and continuous infusion technique, with the former used more often than the latter. In the bolus technique, first, a small volume of ultrasound contrast agent (UCA) is quickly flushed into the patient through a peripheral vein. The UCA can be visualized a few seconds after intravenous administration, by means of contrast specific imaging technique. A single plane is imaged at 10-20 frames per second and the duration of contrast enhancement varies from 3-5 minutes depending on the dose and type of UCA. After the bolus injection, the wash in and wash out of the UCA through region of interest (ROI) is captured and time intensity curve (TIC) is derived from the average intensity shown as a function of time (figure 7)[56].

In comparison, the disruption-replenishment technique is performed during a continuous infusion of contrast agent via an infusion pump for 5-20 minutes. Microbubbles are initially imaged at low MI to avoid disruption but then can be disrupted at regular intervals by increasing the MI. With replenishment times for most tissues ranging between 5-10 seconds, flow measurements can be repeated many times and multiple imaging planes can be visualized during a single scanning session [58].

The contrast bolus technique has the advantage of being a quick and easy means of acquiring information regarding different blood flow parameters such as relative flow velocity, and relative vascular volume. On the other hand, the infusion technique needs more contrast and expensive equipment, although it has the advantage that more than one plane can be scanned at a time[59].
Based on the above reasoning, contrast bolus technique is adopted in this thesis. After administration of the contrast bolus injection, when the microbubbles reach the region of interest within the liver, different parameters are derived from the time intensity curve (TIC) (figure 7) [60].

Figure 7. The contrast bolus injection technique. The Graph shows the TIC derived from microbubble ultrasound. Adapted from [14]. $I_p$: Peak Intensity $t_0$: time at initial (zero) intensity $t_p$: Time at peak intensity $I_0$: Initial Intensity WIT: wash in time MTT: Mean transit time AUC: area under the curve AIU: arbitrary international units.

These are:

- **Time to peak** is the time from zero to maximum backscattered ultrasound signal intensity.
- **Wash-in time** is the time taken from 5% to 95% backscattered ultrasound signal intensity.
Wash-out time is the time taken to return from the peak of the TIC curve to the zero value.

Mean transit time describes the average time that contrast microbubbles take to pass through the ROI, which can be any vessel, tumour or normal liver parenchyma.

Peak intensity is the maximum ultrasound signal intensity in the TIC curve. It indicates regional blood volume.

The area under the curve is the shaded area under the TIC, calculated numerically between the time \( t_0 \) and \( t_{\text{end}} \). This parameter is also related to blood volume.

In ultrasound image intensity, \( I(t) \), is proportional to concentration \( c(t) \),

\[ I(t) = \alpha * c(t) \]

Where \( \alpha \) is a proportionality constant [60].

Thus, by measuring the average intensity in a ROI, and calculating the various blood flow parameters of TIC, relative measures of flow rate in a ROI can be determined [60].

2.1.7 Techniques for compensation of respiratory motion

Various mathematical and empirical models [lognormal wash in wash-out curve (LNWIWO), gamma variate function, local density random walk model (LDRW)] developed from the indicator dilution theory are then fitted to microbubble ultrasound derived TIC to create smooth curves and remove noise due to motion of region of interests. They also remove the primary pass, and help in extracting consistent and reproducible blood flow parameters. The lognormal function and LDRW are considered to be better models of fitting these curves, as they take into account the architecture of micro-vasculatures [61].

However, these functional parameters of microcirculation using microbubble ultrasound could be impaired by artefacts. The movement of the scan plane due to respiratory motion is one of the main causes of artefact in quantification, a problem made more severe when
the ROI is small and completely moves out of the plane. Also the TIC of smaller ROI have more pronounced oscillations, which can make accurate measurement of parameters such as RT, PI or AUC very difficult. Also, respiratory motion is not coplanar with the scan plane, and the TIC actually presents information on different planes of the lesion at different times\cite{14}. Although the operator can compensate for respiratory motion using visual diagnosis, this is still the main source of error during quantification of the desirable image data\cite{14}.

Moreover, techniques for compensation of motion of the ROI are inherently flawed. Although research into respiratory gating methods in MRI and radiotherapy has been ongoing for several years, they have not been investigated in relation to abdominal contrast US examination. For respiratory motion compensation, three different techniques have been suggested. First, the breath holding strategy entails instructing patients to hold their breath during acquisition; however, requiring patients to hold their breath in acquisitions of $>20$ sec is found to be problematic\cite{62}.

Second, registration strategy is a technique in which images of dynamic sequence are aligned, although this method has limited applicability due to technical issues with abdominal contrast ultrasound, which include non-uniform uptake of contrast and complicated organ motions in terms of deformation and amplitude\cite{63}.

Finally, gating strategy, which has been employed in this thesis, does not entail patients being given any instructions for breath holding; instead they are required to breathe normally. The frames at extreme phases of the respiratory cycle which are deviant from the
reference position of the diaphragm are removed, either manually or by automatic filtering (explained in chapter 3 page 78 )[64].

With the advent of new forms of quantification softwares, imaging has become increasingly quantitative in nature, especially when contrast agents are used to probe tissue function for objective evaluation of patients [65], and more work is being done to explore the role of imaging as a biomarker.

2.2 Imaging as a biomarker in cancer treatment

According to the United States Food and Drug Administration (FDA) definition,

“A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention”[66].

“An imaging biomarker can be defined as anatomic, physiologic, biochemical, or molecular parameters detectable with imaging methods used to establish the presence or severity of the disease [67]”.

There is currently much focus on the use of imaging biomarkers as surrogate endpoints. The term surrogate comes from the Latin word ‘surrogatum’ meaning substitute. In clinical trials, a surrogate endpoint can substitute for an established clinical endpoint, i.e. it is expected to predict clinical benefit based on epidemiologic, patho-physiologic or other scientific evidence [65]. Meanwhile, a clinical endpoint is a characteristic that helps to measure how a patient feels, functions or survives [68]. Finally, the RECIST criteria are a surrogate endpoint, as compared to progression free survival and disease free survival.
Imaging methods of recording the disease course or assessing therapeutic interventions have a number of advantages [69]. Measurements can be taken non-invasively and repeatedly and can be performed on individuals before and after treatment, thus allowing the evaluation of treatment with respect to a before treatment reference state and reducing inter-individual variability to a great extent [65]. Images can also capture the heterogeneity of the tumour and its response to treatment [70].

The development of ‘targeted biological agents such as bevacizumab, and cetuximab’ has increased the significance of imaging biomarkers. As the overall survival benefits with antiangiogenic therapy and chemotherapy combinations or alone are insignificant [71], finding predictive markers for selection of patients has become critical. Hence, biomarkers could be useful in deciding the optimal schedule and doses of antiangiogenic and cytotoxic chemotherapy combinations [72]. Antiangiogenic therapy, besides having its own unique adverse effects, can also increase the toxicity of chemotherapeutic agents, whereas these toxicities could be identified by biomarkers [73]. To ensure more appropriate and cost-effective use of antiangiogenic agents, it is important to understand the role of angiogenesis in development of liver tumours.

2.3 Angiogenesis and antiangiogenic therapy

Angiogenesis plays a key part in the growth and spread of a tumour [74, 75]. Solid tumours formed as result of angiogenesis are composed of various cell types, such as cancer cells, vascular endothelial cells, and host (stromal) cells. These are embedded in the tumour interstitial matrix (TIM), and are composed of proteins secreted by host cells and tumour
cells, whilst, in addition, they contain proteins leaked from nascent, relatively permeable blood vessels.

Many factors contribute to increased vascular permeability in tumour cells, including wide interendothelial junctions, increased number of fenestrations, vesicles, vesicovacuolar channels in tumour vessels, lack of normal basement membranes and reduced number of pericytes [76]. Pericytes are contractile cells located within the basement membrane of capillary and post capillary venules. They stabilise the vessel wall, controlling endothelial cell proliferation, and thereby the growth of new capillaries[77]. Abnormalities in tumour vasculature and viscosity result in increased resistance to blood flow. As a result, the overall perfusion rate (blood flow rate per unit volume) in a tumour is lower than in normal tissue. The tumour blood flow is also temporally and spatially chaotic, which leads to heterogeneous vascular permeability and a microenvironment.

Tumour vascularity is characterized by several physiological parameters [78], which can be used to calculate the distribution of contrast agents over time. They are suitable for monitoring the effects of cytotoxic chemotherapy as well as antiangiogenic therapy [79].

Clinical parameters describing tumour vascularity [80] include:

- **Blood volume**: Quantity of blood that occupies a given mass of tissue or region of interest (ml or cm³)
- **Blood velocity**: Directional speed of the blood [distance/time], (mm/s)
- **Blood Volumetric Flow**: volume of fluid passing through surface per unit time
Figure 8. Blood volumetric flow. Adapted from [81]

A  Cross sectional area of blood vessel

P1  Pressure at high gradient

P2  Pressure at low gradient

V  \( \text{Velocity} = \frac{\text{Length}}{\text{Time}} \)

F  \( \text{Flow} = \frac{\text{Volume of fluid}}{\text{Time}} \)

Volume of a cylinder = Area x Length

So flow  = \( \frac{\text{Area x Length}}{\text{time}} \)

Velocity  = \( \frac{\text{Distance or Length}}{\text{time}} \)

So flow = Cross Section Area (A) x Velocity (v) = ml / min
In the past, cross sectional area and velocity of hepatic blood vessels have been determined by Doppler ultrasound to describe blood volumetric flow [82, 83]. The rate of blood flow (F), is a function of pressure difference (ΔP) and flow resistance (R) in blood vessels and is determined as $F = \frac{\Delta P}{R}$. Difference in pressure $\Delta P = [P1 - P2] = [R \times \text{Flow}]$. R depends on the vascular architecture (vessel number, length, diameter, branching pattern and organization) and blood viscosity [84]. Vascular architecture is different in tumour blood vessels as compared to normal blood vessels.

### 2.3.1 How do tumour blood vessels differ from normal blood vessels?

There is a marked difference in the way that vessels develop in normal and tumour tissues (figure 9).

![Vascular system of normal tissue](image)
Red represents well-oxygenated arterial blood, blue represents poorly oxygenated venous blood, and green represents lymphatic vessels. Adapted from [86]. In healthy tissue, vascular development is usually well organized as compared to disorganized abnormal and chaotic vascular structures in tumours. In tumours, excessive irregular branching and tangled arrangements of vessels often result in abnormal vascular linkages, with connections between adjacent arteries and arteriovenous shunting. This results in increased resistance to blood flow, which is also contributed to by compression of vessels by tumour cells (59). Tumour blood vessels have more blind vascular endings, with dilated tortuous and chaotic arrangement, while, microscopically, newly formed malignant vessels often have incomplete walls with gaps between adjacent endothelial cells [86]. This results in abnormal vascular permeability and often poorly regulated flow with low vasomotor tone. Due to these factors, the spatially and temporally diverse blood flow in solid tumours leads to a compromised metabolic microenvironment as compared to the efficient, regulated blood flow of normal organs and tissues [87]. Also, low oxygen levels in the tumour microenvironment lead to hypoxic drive and release of pro-angiogenic factors, resulting in increased tumour growth and metastatic potential [88].
2.3.2 Antiangiogenic therapy

Compounds that disrupt existing vessels (vascular disrupting agents) or affect new vessel formation (antiangiogenic) offer potential for anticancer therapy [89]. Antiangiogenic therapy (table 4) has been shown to have activity as single agents, such as sunitinib in renal cell carcinoma, as well as in combination with cytotoxic chemotherapy[90]. The major target of current antiangiogenic therapy is the VEGF pathway, which promotes endothelial proliferation and survival, thereby influencing tumour angiogenesis and vasculogenesis. It is also known as a vascular permeability factor, causing tumour blood vessels to become leaky, and consequently leading to interstitial hypertension (ISH).

<table>
<thead>
<tr>
<th>Agents</th>
<th>Molecular target/pathway</th>
<th>Action</th>
<th>Biological Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal antibodies blocking growth factors or receptors</td>
<td>VEGF</td>
<td>Involved in angiogenesis</td>
<td>Bevacizumab</td>
</tr>
<tr>
<td></td>
<td>EGFR (receptor HER-1)</td>
<td>Plays role in proliferation, angiogenesis, invasion, metastasis, and evasion of programmed cell death</td>
<td>Cetuximab</td>
</tr>
<tr>
<td>Tyrosine Kinase Inhibitor (TKIs)</td>
<td>C-Kit, VEGFR, PDGFR,</td>
<td>Involved in cancer cell proliferation, and evasion of programmed cell death</td>
<td>Sorafenib</td>
</tr>
<tr>
<td></td>
<td>c-Kit</td>
<td>Involved in cell proliferation and differentiation</td>
<td>Imatinib</td>
</tr>
</tbody>
</table>

Table 4. Biological agents and molecular targets. Due to recent advances in molecular biology, many new cancer cell pathways have been discovered. The main classes of biological agents targeting these pathways include PI3K/Akt/mTOR pathway inhibitors, drugs interfering with EGFR or KIT receptors, antiangiogenic drugs and antivascular agents. Adapted from [91, 92].

Antiangiogenic therapy helps in pruning of immature tumour vessels, whilst normal tissue vessels are spared, in a phenomenon known as normalisation [91]. For this reason, the
vascular normalisation window (figure 10) has been suggested in many preclinical and clinical studies.

**Figure 10. Normalisation window:** Cancer cells may be more susceptible to cytotoxic chemotherapy combinations during normalisation. Treatment with antiangiogenic drugs in well defined doses and combinations has been hypothesised to prune immature and dysfunctional blood vessels, without any major damage to normal tissue vasculature, which may improve the delivery of systemically administered cytotoxic agents. Alternatively, if antiangiogenic drugs are administered in a high dose, there will be heavy pruning, resulting in vascular shutdown, so a critical balance is the key to achieving efficient delivery of cytotoxic chemotherapy when combined with antiangiogenic drugs. Adapted from[91].

In addition, antiangiogenic therapy targeting VEGF helps in decreasing ISH, which allows more efficient delivery of cytotoxic chemotherapy [91]. Antiangiogenic therapy also results in induction of cancer cell apoptosis and a decrease in the number of blood circulating endothelial cells [92] Based on preclinical models, antiangiogenic therapy (e.g. bevacizumab) has been proposed to have multiple antitumour effects [93], with regression
of existing tumour vessels and ultimately reduction of tumour size. O’Connor (2009) observed a significant reduction in vascular volume and density within 48 hours of bevacizumab infusion in preclinical tumour models [94], whilst Chris Willett (2004) showed that treatment of rectal cancer with bevacizumab reduced tumour blood volume and perfusion, leading to blanching and shrinkage of the tumour [95].

Within 24 to 48 hours of antiangiogenic therapy, reduced perfusion of the vessels was observed on micro-computed tomography. Meanwhile, using contrast ultrasound, patients with rectal cancer and liver metastases were found to have reduced tumour blood volume within 24 hours of antiangiogenic therapy [95].

Many cytotoxic chemotherapies (e.g. cisplatin) and biological agents (e.g. cetuximab) also have antiangiogenic effects, although developed originally for reasons other than inhibition of tumour angiogenesis [96, 97].

2.3.3 Cytotoxic chemotherapy and antiangiogenic effects

The main target of cytotoxic chemotherapy is proliferating and rapidly dividing tumour cells; however, endothelial cells involved in angiogenesis are also damaged, undergoing spontaneous cell death (apoptosis) that leads to vascular damage. This antivascular effect as a result of endothelial cell killing has not been generally appreciated, mainly due to the rest period between cycles of chemotherapy, which is presumed to repair damage to the vessels[98]. As compared to dose intense chemotherapy, metronomic chemotherapy has been suggested to have both anti-tumour as well as antiangiogenic effects as it is given more frequently in low doses. Frequent weekly schedules prevent the repair of endothelial
cells, so giving the additional benefit of antiangiogenic effect, a phenomenon which has been particularly acknowledged in weekly cycles of taxanes chemotherapy in breast cancer treatment [99].

2.3.4 Selective Internal Radiation Therapy (SIRT)

In the mid-1990s the use of microspheres containing yttrium$^{90}$ gamma particles was suggested for delivering high-dose internal radiotherapy to liver tumours [100]. Yttrium$^{90}$ emits $\beta$ rays with a half-life of 64 hours and average energy of $\leq$1 million electron volts (MeV). A total absorbed radiation dose of 200-300 Gy, with 94% of the radiation received in the first 11 days, is delivered to liver tumours [101]. Ionization radiation interacts at the cellular level with different cellular components such as de-oxy ribonucleic acid (DNA), ribonucleic acid (RNA) and proteins, and this leads to formation of free radicals such as super oxide and hydrogen peroxide. These free radicals cause DNA damage which eventually leads to radiation induced cell killing [102]. Besides inducing cell killing, this type of radiation therapy has been described to have a direct effect on tumour vasculature [103], through the radiation inhibiting ongoing angiogenesis without affecting the established vasculature. In this regard, Geng et al. (2001) demonstrated in veterinary models that treatment with 6 Gray (unit of absorbed radiation) caused a decrease in tumour vasculature and blood flow time within a 72 hour period [104]. This treatment, therefore, offers a dual benefit of micro-embolisation and high-dose radiation induced cell killing. SIR-spheres microspheres, when used in combination with chemotherapy or alone, provide an improvement in liver tumour control by shrinking the tumour and delaying progression in the liver[105]. As there is arterial shutdown but no immediate cell killing, current response
assessment methods are limited; however, functional imaging for assessment of tumour perfusion may offer an effective alternative for this purpose.

Following radioembolisation, about 80% of patients develop a mild fever which settles down within a few days without any treatment. Also approximately 50% of patients complain of abdominal pain and discomfort which settles within 24 hours with routine analgesics, whilst post treatment lethargy and fatigue occurs 1 to 8 weeks after treatment and can last up to 10 days. Mild nausea (40-50% cases) is most common in patients who have received chemotherapy previously and usually settles down within 24 hours with prophylactic antiemetics[106].

2.3.5 Assessment of vascularity and angiogenesis, significance and challenges

Assessment of tumour vascularity and angiogenesis is important for many reasons[107]; in particular, because vascular invasion is a major route of spread for tumours. Hence, assessment of vascularity may help in defining tissue characterization and grade of tumour (low grade vs. high grade or benign vs. malignant), which not only correlate with malignant potential, but also predisposition to metastasize [108]. Also, as blood flow changes occur prior to morphological changes in the tumour, angiogenesis imaging could potentially have a role in detection of occult malignancy, and early response assessment in patients receiving antiangiogenic therapy (69). However, the two main hurdles in further development of antiangiogenic agents are the lack of proper understanding of their mechanisms of action and development of biomarkers to monitor their effects, which are the key topics addressed in this thesis. Understanding liver haemodynamics can help in identification and development of early surrogate imaging biomarkers.
2.4 Liver haemodynamics

Blood flow to the liver is derived from two main vessels, the portal vein and the hepatic artery. The hepatic artery accounts for 25 to 30 percent of total hepatic blood flow, while the portal vein, supplies 70 to 75 percent of the total hepatic blood flow [1].

Previous studies have shown that with the development of metastases in the liver, the proportion of blood supplied by each vessel changes, as metastatic liver tumours receive around 90% of their blood supply from the hepatic artery, resulting in hyper-arterialisation, while normal liver parenchyma continues to receive the majority of its blood supply through the portal vein [109].

For example, Breedis and Young (1954) injected colloidal pigments into the hepatic artery and portal vein during autopsy to assess liver tumour circulation in thirteen human subjects with metastatic carcinomas, concluding that metastases derived between 80% and 100% of their blood supply from the hepatic artery [2].

Liver haemodynamics is characterized by many physiological variations associated with arterial and portal venous blood flow, such as post prandial variability [110].
2.4.1 Post prandial variability and liver homeostasis

Studies have revealed that ingestion of food leads to marked vasodilatation, and consequently increased blood flow in the portal vein, known as postprandial hyperaemia. This increase in portal venous flow after a meal ingestion (i.e. post prandial hyperaemia), which is documented in several human studies [111], leads to splanchnic vasodilation as indicated by the reduced resistance index ($Resistive\ index = \frac{\text{resistance to blood flow measured as peak systolic velocity - end diastolic velocity}}{\text{peak systolic velocity}}$) in the superior mesenteric artery, which then results in an increase in the diameter ratio and cross sectional area of the portal vein [82]. In the post prandial state the increase in portal venous flow significantly alters the liver’s haemodynamics [112].

However, several studies indicate that total hepatic blood flow remains relatively constant regardless of increase in portal flow and decrease in hepatic arterial flow, in different physiological conditions [113]. It has been suggested that a regulatory mechanism is in force which works to ensure homeostasis of the hepatic blood flow. The two vessels (portal vein and hepatic artery) act in a reciprocal fashion such that when blood flow increases in the portal vein, it decreases in the hepatic artery [114]. The reverse happens when the blood flow increases in the hepatic artery, and a corresponding reduction in portal flow has been demonstrated in a few studies [115].

In this regard, a non-invasive Doppler study by Dauzat et al. (1994) revealed that meals induce marked vasodilation of the superior mesenteric artery, followed by increased portal venous flow and reciprocal hepatic artery vasoconstriction [116]. A further study,
conducted by Payen et al. (1990), used Doppler probes directly to measure blood flow in the hepatic artery and portal vein during human liver transplantation. It was found that portal venous clamping led to a sharp increase in arterial blood flow, while clamping the hepatic artery did not produce any significant changes in portal venous flow[117].

Many hypotheses have been put forward in terms of hepatic blood flow homeostasis, and the reciprocal working of the hepatic artery and portal vein. The most widely accepted explanation is the hepatic artery buffer response theory developed by Lautt et al. (1991) [118]. Adenosine, a potent vasodilator present around the arterioles and portal venules, has been suggested as the mediator for this hepatic artery buffer response. It is postulated that in normal conditions, the portal venous flow washes out the adenosine, and thereby reduces the amount of adenosine, consequently leading to vasoconstriction of the hepatic artery [116]. Kawasaki et al. (1990) identified many regulatory mechanisms (intrinsic and extrinsic) around portal and hepatic vasculatures, which govern the changes in hepatic blood flow [114]. Intrinsic regulatory mechanisms include autoregulation (hepatic artery vasoconstrictions due to myogenic response of the arteriolar smooth muscle due to increase perfusion pressure), metabolic controls (such as arterial hypoxemia, hypercarbia, and alkalosis), and hepatic arterial buffer response, whilst extrinsic regulatory mechanisms include neural control through branches of the vagus, splanchnic and phrenic nerves, and humoral factors such as epinephrine.
2.4.2 Measurement of liver haemodynamics

For measurement of liver haemodynamics and to monitor postprandial haemodynamic changes, many techniques have been used extensively in the past. Among these, thermal dilution [119], microsphere [120] and Doppler ultrasound [121] techniques have been used in demonstrating an increased portal venous blood flow postprandially (after ingesting food) in animals, while in humans, clearance methods for evaluating hepatic blood flow (arterial and portal blood flow) [122] and labelled albumin [123] or Doppler ultrasound [124] for evaluation of portal venous flow have been described in the literature.

Indocyanine green infusion technique has also been used for measurement of liver haemodynamics, although its usefulness is limited in severe liver disease due to inadequate clearance of the dye [125]. This technique describes the rate of extraction from the bloodstream of an indicator substance exclusively removed by the liver as a function of the liver blood flow[126]. Indocyanine green is used as an indicator as it has little extra-hepatic uptake and negligible extra-hepatic metabolism. After continuous infusion through peripheral intravenous route, the infusion rate (I) for inulin is equal to the hepatic extraction rate (E) when it is such that the concentration of the indicator in the peripheral blood is constant. The limitation of this technique is that it can only estimate total liver blood flow, and its accuracy may be affected by extrahepatic shunting and alteration in hepatic clearance in the diseased liver[127].
2.4.3 Doppler Perfusion Index (DPI)

The Doppler perfusion index has been proposed as a measurement of hepatic arterialisation to predict the presence of liver metastases and is defined as the ratio of the hepatic arterial volume flow ($FV_{ha}$) to the sum of the hepatic artery and portal vein volume flow ($FV_{pv}$) [4].

$$DPI = \frac{FV_{ha}}{FV_{ha} + FV_{pa}}$$

Volumetric flow is calculated by multiplying the mean velocity of blood by the cross sectional area of the vessel.

$$Flow = \frac{Volume \ of \ Fluid}{Time} = \text{Velocity} \times \text{Vessel Cross section area} (A) = V \times A \ \text{ml / min}$$

Leen et al., in 2000, were the first to suggest that DPI (ratio of volumetric flow hepatic artery to volumetric flow of the portal vein and hepatic artery), can be used to correctly identify colorectal cancer (CRC) patients with high risk of recurrence, so that they can receive appropriate cytotoxic chemotherapy to reduce the risk of recurrence, post operatively. In their study they found that at a 5 year follow up, patients with high $DPI > 0.30$ had disease free survival (DFS) of 22% and overall survival (OS) of 29% compared to patients with normal DPI, who had 89% DFS, and 91 % OS [7]. The possible impact of this study could be in better selection of high risk early stage patients in adjuvant settings, who should receive chemotherapy to improve DFS and OS. Meanwhile, patients with low risk of recurrence based on $DPI <0.30$, could be spared from toxic side effects of unnecessary cytotoxic chemotherapy, which can also help in saving costs.
After determining that high DPI >0.30 was associated with poor outcome, Leen et al.(2000) investigated the impact of removing the primary tumour on DPI to find out whether the primary tumour is responsible for producing these blood flow changes [128]. Although patients who had raised DPI before surgery recorded reductions in values, these still remained in an abnormal range according to results of the previous experiment (i.e. >0.30). They hypothesised that rather than affecting the primary tumour, micrometastases in the liver altered the hepatic haemodynamic, which was reflected in the DPI [128].

DPI might be useful in distinguishing between patients that should accept the toxicity of treatment with cytotoxic chemotherapy, because of potential benefit due to occult metastases, as opposed to those unlikely to benefit, because of low risk of metastases. Meanwhile, in surgical practice, DPI may be helpful in selection for extensive cure-attempting resection, especially among elderly and high risk patients [129]. The lower cost of DPI compared to other imaging modalities should reduce the economic cost of following-up patients to identify who may be developing liver metastases [130].

Unfortunately, there are many issues with DPI measurements. DPI measurements are technically difficult and time consuming to perform (due to issues with beam angles and inaccurate measurement of the areas of the vessel studies) and are strongly operator dependent. Leen et al.’s results could not be reproduced by other groups [131, 132], whilst about 30% of patients have variations in hepatic artery anatomy [7, 133]. Moreover, the long term results of initial liver blood flow parameters calculated by Doppler studies are yet to be confirmed in patients with liver metastases and primary liver cancers (HCC). Seeing the potential role of ultrasound contrast agents (UCA) in measurement of blood perfusion and
flow volume, in 2002, Ramnarine et al. suggested an index similar to the DPI called the contrast enhanced Doppler perfusion index (CE-DPI) [134].

2.4.4 Contrast enhanced Doppler Perfusion Index (CE-DPI)

Ramnarine et al. administered 0.3-2.4 ml bolus injections of the contrast agent Levovist to patients with liver metastases and haemangiomas and derived time intensity curves (TIC) from the hepatic artery and portal vein by using Doppler [134]. The CE-DPI was defined as the ratio of wash in slope (WIS) derived from TIC of the hepatic artery to the sum of wash in slope derived from the hepatic artery (HA) and portal vein (PV). This was represented as:

$$CE\text{-DPI} = \frac{WIS(\text{HA})}{WIS\text{ HA} + WIS\text{ PV}}$$

They found significant difference in CE-DPI values between tumours (Mean= 0.59 and 95 % confidence interval 0.54–0.63) and patients with haemangiomas (Mean= 0.33and 95% confidence interval 0.24–0.41 $P < .0001$) and recommended further studies to establish the clinical value of CE-DPI as a prognostic biomarker or as a marker for the presence or absence of occult liver metastases[134].

In the same decade other researchers had begun using contrast ultrasound for measurement of perfusion of liver tumours in order to identify various tumour perfusion parameters and use perfusion as a prognostic biomarker.
2.4.5 Tumour perfusion studies

In 2010, Averkiou et al. performed a tumour perfusion study to evaluate early response to cytotoxic and antiangiogenic chemotherapy combinations in CRC patients with liver metastases [14]. They injected an intravenous bolus of 2.0 ml SonoVue microbubbles followed by a saline flush of 5 ml. Using WIT and WIT ratio (ratio of wash-in time of the lesion to that of the normal parenchyma) derived from the TIC of the tumour and surrounding parenchyma to compare perfusion rates, they found that there was an increase in WITR among responders (mean WITR increase of 17% after the first dose of treatment and 75% at the end of the therapy). WITR predicted the response in four out of five patients (80%) responding to the first treatment. WITR therefore may be a new surrogate marker indicative of early tumour response for CRC patients undergoing cytotoxic and antiangiogenic therapy [14].

Lassau et al. have conducted a number of microbubble US studies on tumour perfusion [135]. In one such study, they assessed microbubble ultrasound as a prognostic tool for metastatic renal cell carcinoma (RCC) receiving sunitinib treatment. They derived perfusion parameters extracted from the TIC of the index lesion inside the liver of patients who had received sunitinib, and then assessed correlation between the microbubble ultrasound parameters and RECIST as well as DFS and OS. The interval changes in five of the seven tumour perfusion parameters (PI, TPI, AUC, AUWI, MTT, and RT, AUWO), between baseline and day 15, significantly correlated with RECIST. Two microbubble ultrasound parameters [time to peak intensity (TPI) and wash in slope (WIS) were significantly associated with DFS, whilst TPI was also significantly associated with OS. They concluded that microbubble
ultrasound has a potential role in predicting early response to sunitinib in patients with metastatic RCC [136].

In a further study, Lassau et al. assessed correlation between microbubble US parameters and RECIST as well as DFS and OS in 37 patients with HCC receiving antiangiogenic treatment (bevacizumab) [137]. On RECIST, patients with partial or complete response or stable disease were categorised as responders, whereas patients with progressive disease were defined as non-responders. In this study, they performed microbubble ultrasound scans at day 0, 3, 7, 14 and 60 after treatment. The interval changes in tumour perfusion parameters between day 0 and the other time points were then compared between responders and non-responders. The percentage decrease in tumour perfusion parameters between day 0 and day 3 correlated with tumour response, from which they concluded that microbubble ultrasound can be used to quantify dynamic changes in tumour vascularity as early as 3 days after bevacizumab administration [137].

In a Canadian study, Williams et al. (2011) described a technique for characterizing vascular response of renal cell carcinoma to a targeted therapy such as sunitinib by using microbubble US [138]. They scanned 17 patients with RCC receiving sunitinib, before and 2 weeks after treatment, using bolus as well as disruption replenishment technique. They found evidence of a decrease in fractional blood volume of the tumour of 73.2 % using disruption replenishment technique (explained in chapter 2.1.6, page 38) but could not find a correlation between this decrease and RECIST after a 6 week interval. They concluded that changes in microbubble ultrasound parameters over 2 weeks did not correlate with RECIST.
or PFS, although microbubble ultrasound provides reproducible and sensitive assessment of vascular changes in response to antiangiogenic therapy [138].

In addition, a Japanese group published a study based on quantitative assessment of tumour perfusion in patients with HCC receiving transarterial chemoembolisation (TACE). They found that mean transit time (MTT) and rise time were prolonged by TACE in all 3 patients, and concluded that MTT as a quantitative time parameter may indicate an early response to TACE in patients with HCC [139].

Another research group worked on the feasibility of microbubble ultrasound for early response evaluation in 30 CRC patients with liver metastases who were receiving bevacizumab. These patients underwent microbubble ultrasound at baseline, before and in the 2nd and 3rd cycle of bevacizumab (Avastin) treatment. Three parameters (peak intensity, time to peak intensity (TPI) and rise time (RT)) were correlated with radiological response. Based on these response criteria, there was significant (P < 0.001) correlation of time to peak intensity (TPI) between metastases of responders (9.08 s) and non-responders (14.76 s). In this study, TPI also significantly (P < 0.01) differed between responders and non-responders. In contrast, no significant differences were identified in PI and RT between responders and non-responders. They concluded that microbubble ultrasound might function as a surrogate biomarker to predict treatment response in CRC patients with liver metastases receiving antiangiogenic therapy [140]. A comparison of different studies is presented in Appendix 6.
There are multiple fundamental flaws with these studies. As mentioned earlier in this chapter, liver metastases being supplied by dual blood supply (i.e. hepatic artery and portal vein), there are dynamic shifts in differential distribution of blood flow between these metastases. If we measure perfusion changes across individual liver metastases, there are possible issues over variability and reliability. Also there is a natural selection bias towards measuring perfusion change in larger metastases. In these studies, to monitor response to therapy, perfusion of a single index liver tumour was assessed whereas there are multiple occult micro-metastases in the liver, and these may behave differently in terms of response in quantification of tumour perfusion. To address this problem microbubble scanning can be performed with many boluses of contrast agent. However, in that case, earlier contrast injections have an effect on the subsequent injections due to microbubbles being trapped inside liver sinusoids and thereby producing artifacts. This also leads to increased cost and prolonged duration of the scanning as at least 10 minutes must elapse between subsequent boluses of microbubbles. To overcome this problem affecting tumour perfusion studies conducted in the past, we suggested a response assessment based on total liver blood flow over the entire liver tumour.
2.4.6 Contrast Enhanced Hepatic Perfusion Index (CE-HPI)

In this thesis, microbubble ultrasound is used to develop an alternative index: the contrast-enhanced hepatic perfusion index (CE-HPI), for measurement of blood flow changes in the liver. Following intravenous administration of UCA, liver blood flow is assessed by quantifying the wash-in characteristics of the contrast agent. The CE-HPI is determined by calculating the ratio of hepatic arterial to portal venous volumetric blood flow [134]. The wash-in and wash-out kinetics of contrast agents in an organ or tissue after a bolus injection can be measured quantitatively. This generally entails specifying a region of interest (ROI) and recording total pixel intensity in this region as a function of time; then measurement is carried out off line from the video signal. For a homogenous distribution of MB in the blood, the maximum height of the time intensity curve (peak intensity) is a measure of blood volume (B) in the region of interest, while the wash in slope, which indicates rate of microbubble filling in, is a measure of blood velocity (v) in the region of interest. Blood flow (f) can be defined as the product of mean blood velocity (V) and cross sectional area A (blood volume) [141].

\[
F \sim A \times V
\]

\[
\text{CE-HPI} = \frac{F \times V_{HA}}{F \times V_{PV}}
\]

Contrast Phases in the liver and how CE-HPI works

The dual blood supply to the liver through the hepatic artery and portal vein results in two separate sets of wash-in kinetics (figure 11).
Figure 11. Contrast phases in the liver, as indicated by time intensity curves. Adapted from [142].

Using contrast ultrasound: the red curve derives from the hepatic artery, whilst the blue curve derives from the portal vein. Initially, at time point 10-20 sec, there is contrast enhancement due to wash-in from the hepatic artery (arterial phase). Approximately 30-40 seconds later, contrast enhancement due to wash-in from the portal vein is seen (venous phase). Normal hepatic parenchyma is mainly supplied by the portal vein, and therefore contrast enhancement in liver tissue is substantially stronger in the portal venous phase. In metastases or primary liver cancers the gradient is reversed, because of arterialisation. By measuring this gradient, before, during and after chemotherapy, it may be possible to predict an early response to cytotoxic chemotherapy and biological agents in different tumours.
Chapter 3  Methods

This was a single centre, pilot and feasibility study to prospectively explore the role of microbubble ultrasound derived liver blood flow parameters as early response biomarkers in a heterogeneous population of patients.

The study was conducted in the outpatient department of the gastrointestinal oncology unit at Hammersmith Hospital from May 2010 to July 2012; ethics approval (08/H0711/5) was gained from West London research ethics committee (REC) 2.

Eligible participants had the following cancers accessible to DCE US: histologically confirmed primary liver cancer (HCC) and secondary liver tumours with primaries from colorectal cancer (CRC), pancreatic cancer, cholangiocarcinomas, neuroendocrine tumours (NETs), and gastrointestinal stromal tumours (GIST).

**Inclusion criteria** were:

1. Written informed consent,
2. over 18yrs old,
3. at least one primary or metastatic lesion in the liver >2cm that was accessible by DCE–US,
4. about to start an anticancer therapy (chemotherapy, biological agent or chemo-embolisation or radio-embolisation),
5. Willing and able to comply with scheduled visits, treatment plans, laboratory tests, and other study procedures.
Exclusion criteria were:-

1. Performance Status, eastern cooperative oncology group (ECOG) >2 (defined in appendix 1)
2. psychiatric conditions, including dementia or significant altered mental status,
3. known allergy to SonoVue (contrast),
4. pregnancy or planning to be pregnant
5. Recent acute coronary syndrome, recent symptoms involving the heart or unstable angina or ischaemic cardiac disease, right-to-left shunts, severe pulmonary hypertension, uncontrolled hypertension or adult respiratory distress syndrome.

3.1 Method of recruitment, sample size and gaining patients’ consent

The study participants were screened from the Specialist Liver MDT (multidisciplinary team) clinic and gastrointestinal oncology clinic at Hammersmith Hospital from May 2009 to July 2012, using non-probability consecutive sampling technique. Non-probability sampling includes all available subjects, and therefore the sample is a better representation of the entire population. The drawback is that it does not include randomization, and the sample may or may not represent the entire population accurately.

Being a pilot study, there were no data from previous studies to inform sample size calculations. However, every effort was made to ensure that the sample of this pilot study was representative of the target study population, and that it would be large enough to provide useful information about the aspects that were being assessed for feasibility, and also include sample size calculation for a future randomized study.
Patients were initially screened from the gastrointestinal outpatient clinics at the oncology department, and also identified from liver MDT at Hammersmith Hospital. These patients were then approached on their appointment days, by the author of the thesis. They were informed at length about the whole procedure of this study, including rationale and expected side effects. They were also handed patient information sheets (PIS) which included sections on the purpose of this study, benefits and risks of taking part in the study. Patients were assured that participation in this study was voluntary and would not affect their treatment in any way. It was further explained that they could withdraw from it at any time without giving a reason, and without medical treatment being affected. Patients were given at least 24 hours to decide whether to consent.

3.2 Interventions and treatment regimens

Cytotoxic chemotherapeutic agents and regimens are described in Appendix 5.

3.3 DCE-US schedule

DCE–US was performed at the following intervals,

a) Before treatment (up to 72 hours pre-treatment to fit in with clinic appointments)

b) Two weeks after first day of treatment.

c) At 6 weeks [+/− 2 weeks to fit in with clinic appointment for patients receiving selective internal radiation therapy (SIRT)]

Patients were monitored for any untoward effects in the scanning area for 30 minutes after the completion of contrast ultrasound.
3.4 Imaging Protocol

3.4.1 Operators
Image acquisition was performed by three trained operators (AM, XF, and YZ). XF had 10 years of contrast scan experience and YZ had 7 years’ contrast scan experience. AM, the author of this manuscript, acquired experience of 100 liver scans, under supervision in the radiology department of Hammersmith Hospital from April 2010 to June 2010, before starting work on thesis, and also observed twenty contrast liver scans under supervision of EL at the oncology department, Hammersmith Hospital.

3.4.2 Imaging equipment

3.4.2.1 Contrast Agent
The contrast agent used is SonoVue (Bracco SpA, Milan, Italy), which consists of a phospholipid shell containing the inert gas sulphur hexafluoride.

![Image of SonoVue microbubbles](image)

Figure 12. *SonoVue, consisting of unilayer of phospholipid shell*, containing sulphur hexafluoride (SF6) gas. Sulphur hexafluoride gas has high molecular weight and low solubility which, with the phospholipid shell, give prolonged stability to these microbubbles in the blood. Adapted from [143].
SonoVue microbubbles also have uniform diameter, which improves the backscattering and harmonic behaviour at low acoustic power insonation [144].

Common side effects of the contrast agent SonoVue are headache, nausea, injection site reactions including pain, bruising, burning, and paraesthesia, seen in between 1 and 10 patients in 100 [145]. SonoVue is contraindicated in the following conditions [145, 146]:

a) Patients with recent acute coronary syndrome, ischaemic cardiac, or unstable angina disease, right-to-left cardiac shunts, uncontrolled hypertension, severe pulmonary hypertension or adult respiratory distress syndrome [147]

b) Breast-feeding or pregnant women

c) Patients with hypersensitivity to sulphur hexafluoride [148]

Hence, the patients were duly warned about these conditions and if they were found to have any one of these conditions they were excluded from the study.

3.4.2.2 Method of Preparation of SonoVue: vial preparation

SonoVue, consisting of phospholipid shell and SF6 gas, is prepared as a lyophilised powder [149].

Figure 13. Package of the commercial microbubble based agent SonoVue (Bracco, Milan, Italy).
Lyophilisate powder. The SonoVue kit consists of one vial consisting of 25 mg of lyophilised sulphur hexafluoride gas, 1 pre-filled syringe containing 5 ml of sodium chloride and 1 mini-spike transfer system. Adapted from [143].

Steps for preparing SonoVue are shown in figure 14 below.

**Figure 14. Steps to prepare the SonoVue (microbubbles) vial prior to injection.** The following steps are taken to prepare the vial for SonoVue. 1. Plunger rod is connected by screwing it clockwise into the syringe. 2. Next, the MiniSpike transfer system blister is opened and the syringe tip cap is removed. Then the system cap is opened and connected to the transfer system by screwing it in a clockwise direction. 4. Now the flip cap glass protective disk is removed from the vial. The vial is quickly slid into the transparent sleeve of the...
transfer system and pressed firmly to lock the vial in place 5. The plunger rod is now pushed to empty the contents of the syringe. 6. The whole vial is shaken vigorously for 20 seconds so that all contents of the vial are thoroughly mixed, making a white milky suspension. 7. The plunger with vial is inverted and SonoVue microbubbles are carefully drawn into the syringe. 8. The syringe is now unscrewed from the transfer system and the cap of the vial is quickly closed to stop the gas leaking from the vial. Adapted from [143].

3.4.2.3 Method of injection

Following are the steps for injecting microbubbles prior to the scanning procedure (figure 15)

![Figure 15. Method of SonoVue microbubble injection.](image)

- a. Withdraw 2 mls of SonoVue microbubbles suspension from the vial.
- b. Gently shake it for a few seconds prior to injection, to avoid sedimentation.
- d. Then connect the 3-way stop cock to 16–18 gauge intravenous cannulas. Small (22–24 gauge) intravenous cannulas are avoided, as microbubbles may be destroyed during the injection.
- e. Normal saline is used to flush the line after completion of the scanning procedure. For diagnostic purposes, the microbubbles prepared in this way are used within 6 hours, to prevent degradation with time, which may affect the
quality of diagnostic imaging. For quantification of images, the agent should be mixed within a few seconds before the first injection. Adapted from [143].

3.4.2.4 Pharmacokinetics and Pharmacodynamics of microbubbles
SonoVue microbubbles have 6 minutes elimination half-life. Being an inert gas, sulphur hexafluoride (SF$_6$) gas does not affect the metabolism of the body. About half of the sulphur SF$_6$ gas is released within 2 minutes, while > 80% of it is exhaled through the lungs in eleven minutes. Other components, including the phospholipid shells, are filtered by the kidneys and eliminated by the liver.

3.4.2.5 Ultrasound scanner
We used a mobile IU22 ultrasound scanner (Philips Healthcare, Andover, MA). This machine has the advantage of a C5-1 curvilinear transducer (probe) with a non-linear imaging mode (explained in chapter 2.1.5 page 33) to enable scanning with contrast agent.

3.4.2.6 Ultrasound scanner settings

Machine settings such as mechanical index (M.I), focal depth, dynamic range, overall gain and transducer frequency were kept constant throughout the study (table 5). These parameters are explained below.

Overall Gain

Ultrasound waves are attenuated through the tissues, which results in small amplitude of the returning echoes. Overall gain is the equal amplification of all received signals, to enhance the target clearly before display and enable the image to be seen more clearly. It provides equal amplification of all the echo signals irrespective of their depth and time taken to return to the transducer, also termed linear amplification or overall gain. It
determines the overall brightness of the image: if either too bright or too dark it is difficult
to see subtle differences in structures.

**Time gain compensation**
Ultrasound echoes deflected from deeper structures are attenuated more than the
superficial structures, leading to weak echoes and consequently a weak image. To
compensate attenuation with depth, TGC is used for amplification. In other words, TGC is
the process of amplification of echo signals by compensating the attenuation due to depth,
in a way that echoes from deeper structures are more amplified than echoes from
superficial structures, providing selective gain adjustment at different depths.

**Focus**
Focus is the manipulation of the ultrasound pulse so that it is narrowest at a particular
depth.

**Resolution:**
This is the ability to distinguish or delineate between two subjects. Lateral resolution is the
ability to distinguish between two subjects perpendicular to the ultrasound beam, whilst
axial resolution is the ability to distinguish between two subjects which are parallel to the
main beam.

**Dynamic Range (DR)**
Dynamic range (DR) is the range from the largest echo which does not cause the distortion
of the echo to the smallest echo which is distinguishable from the noise. Logarithmic (non-
linear) amplification of echoes is called Dynamic range compression.
### Control Setting

<table>
<thead>
<tr>
<th>Control</th>
<th>Setting</th>
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</thead>
<tbody>
<tr>
<td>Ultrasound machine</td>
<td>Philips Healthcare, IU22 Andover, MA</td>
</tr>
<tr>
<td>Transducer</td>
<td>C 5-1 curvilinear</td>
</tr>
<tr>
<td>Mode</td>
<td>Contrast specific non-linear pulse sequence (power modulation)</td>
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<tr>
<td>Centre frequency</td>
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<td>Mechanical index</td>
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<td>Side by side contrast / B mode</td>
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<tr>
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<tr>
<td>Video Loop length</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Quantification software</td>
<td>QLAB software (Philips Health care, Andover, MA)</td>
</tr>
</tbody>
</table>

Table 5. Summary of machine settings for contrast specific imaging in this thesis. The same settings were repeated precisely at all follow-up scans.

### 3.4.3 Imaging protocol for the evaluation of liver blood flow parameters

The following protocol is adopted in the thesis for the evaluation of liver blood flow using microbubble ultrasound [14, 150].

1. **Before starting**, confirm that the Informed consent form has been signed by the patient and the patient understands the whole procedure. Examine baseline image and make sure all subsequent images are in the same plane.
b) Ensure patient preparation and fasting for 4 hours at least prior to each DCE-US exam. Patients are allowed to drink water. Try to keep the patient as calm as possible as a relaxed patient is extremely important for the collection of a good loop. No advice is given to the patient regarding breathing, to achieve a natural sinusoidal breathing rhythm in the captured loops.

c) Insert patient name on Philips iu22 monitor and select C5-1 transducer and general abdomen category on the Philips machine monitor.

d) Use inter-costal space approach to find portal vein and hepatic artery as regions of interest. Stay in sagittal plane with gentle breathing (no breath holding). Switch to transducer knob and select default setting for contrast mode and switch to side-by-side

e) An 18-20 Gauge intravenous cannula is inserted in the ante-cubital fossa vein and secured with tape with 3 way-taps. Long tubings should be avoided because they increase the dead space (section 3.4.2.3).

f) Prepare SonoVue according to instructions (section 3.4.2.2). Two ml of SonoVue bolus is drawn from the vial, after careful agitation, and then injected directly to IV line.

g) Timer on the machine is started and the whole loop is captured for 1 minute. Stay in position for the 1 minute video loop acquisition. After finishing the loop, 5-ml normal saline is used to flush the line. After 10 minutes, repeat the whole procedure with the remaining 2mL of SonoVue.
h) The loops are acquired in the same plane, every time the patient is scanned. Rib shadowing is avoided and out of plane motion is minimized by holding the probe steady to improve the quality of image and quantification of analysis.

3.4.4 Safety Monitoring
After scanning, patients are monitored and kept under observation for any hypersensitivity reactions in the outpatient department treatment area for 30 minutes, which is provided with emergency trolleys. They were pre-warned to report about any reactions and unusual symptoms such as chest tightness, wheezing, dizziness, shortness of breath.

3.4.5 Image transfer
A maximum of 1 minute contrast/B-mode side-by-side cine loop is recorded, with data acquisition starting immediately after the contrast bolus injection. These cine loops are then transferred to a personal computer for data analysis.

The data are saved in folders with the following naming scheme: year (yyyy) month (mm) day (dd). For example, a study performed on March 15, 2012 is saved in a folder named 20120315. Any data (loops) that are problematic are removed from the folder.

The one minute loop includes wash in parameters, such as wash in time, peak intensity, and rise time, and avoids recirculation, which starts at around 40 seconds, and complex wash out parameters like wash out time, area under the curve. Respiratory motion of the patient and the transducer motion associated with the technique makes it technically difficult to maintain the same image, for image loops with duration of more than 1 minute. Image
analysis and quantification was done weekly by the author of the thesis, for all the cases scanned during that week.

3.4.6 QLAB Commercial quantification software

QLAB software version 6 (Philips Health care, Andover, MA) is a software application package, which is designed for the on-line and off-line review and quantification of ultrasound imaging studies. This software allows for manual region of interest (such as hepatic artery, portal vein, normal liver parenchyma, liver tumours) selection, measurement of the selected ROI area, and provides linear (logarithmic compression removed) data for the time intensity curves. It is also useful as a means of exporting the data in a form accessible to the end user [151].

3.5 Image analysis:

A. Initial steps and important functions on QLAB Software

Initial steps and important functions on QLAB software have been explained in Appendix 2 and 3.

B. Respiratory gating technique

The time intensity curves TICs derived from the ROI are associated with many fluctuations. The hepatic artery and portal vein lumens are very small, so regions of interest covering them fall out of plane with the respiratory phases (inspiration and expiration). Therefore, respiratory gating is performed to get smooth curves. Following are the steps for respiratory gating.
Figure 16. Step 1: Identification of bright reflector (generally diaphragm) on brightness mode.

Observe the grey scale image to identify which bright reflector moves synchronously with the breathing, to be used for respiratory gating. The diaphragm is the ideal reflector.

Figure 17. Step 2: Selection of diaphragm and region of interest:

Go to estimated peak of arterial enhancement (generally at about 10-12 seconds after contrast bolus injection) and select the position of bright reflector (diaphragm) with the ROI (such as hepatic artery or portal vein) in the desired position; it could be either end expiration or end inspiration.
Figure 18. Step 3: Drawing region of interest (shown in red on brightness mode around the diaphragm): Draw a ROI on diaphragm in the fundamental image to observe respiratory motion.

Figure 19. Step 4: Time intensity curve for diaphragm rhythmic movements: the resultant TIC should be sinusoidal.

Step 5: Select a reference frame which contains the vessel active filter function, as during most of the breathing cycle, the region of interest is not in the desired frame, rather at undesired peaks and out of frame. By selecting a reference frame which contains the vessel of interest (identified on the B-mode or the contrast image) and deleting all frames where the vessel of interest is not visible because of out-of-plane motion, approximately 30% of all frames in both the hepatic artery and portal vein image loops are deleted. Because out of-plane motion could happen at different time points for the hepatic artery and portal vein, respiratory gating to quantify contrast uptake is performed separately in the hepatic artery...
and the portal vein, creating two different sets of frames and resulting in derivation of separate time intensity curves from the hepatic artery and portal vein.

**Step 6:** Once the unnecessary frames are removed, decide whether the gating is satisfactory. If not satisfactory, all the frames should be brought back, and the procedure tried again.

**Step 7:** Once gating is performed, draw the region of interest around the tumour / hepatic artery / portal vein. If there are no problems with the loops, familiar wash in- washout bolus time intensity curves should be found.

**Step 8:** Motion compensation (a feature given in QLAB software) could also be tried. Resultant correction should be inspected and small adjustments can be made by using “create key or main frames” function from the left menu of the QLAB software.

**Step 9:** Curve fitting by using local density random walk model (LDRW) or log normal wash in wash out (LNWIWO) curves, and important features can be noted after curve fitting.

**TIC without respiratory gating**

Red: TIC derived from hepatic artery

Blue: TIC derived from portal vein

*Figure 20 A. Curves without respiratory gating*
Figure 20 B. Step 6: comparatively smooth curves after respiratory gating

**Step 10:** The following parameters are measured, from the TIC, after respiratory gating and applying the LDRW model or LNWIWO curve models.

Once PI and WIS are derived from HAP and PV TICs, the contrast-enhanced hepatic perfusion index (CE-HPI), which reflects the ratio of hepatic arterial to portal venous blood flow to the liver, is defined as:

$$\text{CE-HPI} = \frac{WIS(HA) \times PI(HA)}{WIS(PV) \times PI(PV)}$$

A digital video disc (DVD) with recorded video loops of 2 scans with and without respiratory gating and quantification are attached.
Figure 21. Example of the smoothened time intensity curves derived from hepatic artery and portal vein, after respiratory gating.

This figure is showing

(a) Intercostal plane of the porta hepatis, including the portal vein blue curve (PV), the hepatic artery yellow curve (HA), which is adjacent to the portal vein but not visualized before contrast injection

(b) The image shows the hepatic artery at 7.34 s post contrast injection, with enhanced signal well before the portal vein.

(c) At 16.04 s, the portal vein shows a contrast signal.

(d) Calculated TIC: motion-dependent changes of the corresponding ROI are corrected and the curves are smoothed by the software.
3.6 Image analysis audit

Image quality and quantification analysis by respiratory gating was audited by MA, who has 10 years of experience in quantification analysis of imaging. MA was blinded to the clinical information and RECIST.

3.7 Follow up analysis

Data were transferred to an excel spread sheet for calculation of liver blood flow parameters. Percentage changes in mean / median values at 2 weeks intervals were calculated and compared for early response assessment.

3.8 Outcomes

The RECIST criteria 1 was used to define complete response (CR), partial response (PR) or progressive disease (PD) based on CT scan performed before treatment and post treatment at 3 months. RECIST was performed by independent assessors (radiologists) blinded to the results of ultrasound scans. Patients were categorised as good responders (partial response), bad responders (SD) and non-responders (PD).

Secondary end points PFS and OS for the patients were also determined and correlated with blood flow parameters, including CE-HPI. PFS was defined as the time from randomization to the date of disease progression or death, whichever occurred first. Overall survival (OS) was defined as the time from randomization to the date of death from any cause.
3.9 Statistical Methods and Data Analysis

Statistical analysis was performed with spreadsheets (Excel 2007, Microsoft, California, USA) using GraphPad Prism 5.01, GraphPad Software, San Diego, CA. The results were reported as the mean (SD) for parametric data and median, interquartile range (IQR) for non-parametric data. Statistical significance was set at p < 0.05. The 95% confidence intervals were calculated.

Intraclass correlation coefficients (ICCC), coefficient of variations (COV) and Bland-Altman charts were plotted for reproducibility and the coefficient of variation (SD/mean) was calculated as a measure of variability.

An ICC is defined as “A relative measurement of reproducibility, in which variation due to measurement error (difference between the actual value and the measured value of a variable) is compared with the variation between the subjects”[152]. The ICC value of 0.2 indicates poor agreement: 0.3-0.4 indicates fair agreement; 0.5-0.6 indicates moderate agreement; 0.7-0.8 indicates strong agreement; and >0.8 indicates almost perfect agreement[153].

In COV, reproducibility is described as “the variation between measurements in relation to the mean value of all measurements, and determined by the ratio of the standard deviation (a measure of the dispersion of a set of data from its mean) as a percentage of the mean value of the variable” [152].

In a Bland-Altman plot “the difference between the two measurements per subject is plotted against the mean of the two measurements, assuming that the mean difference between two arbitrary observers is 0 [152]. The limits of agreement are then defined as
−1.96 SD and +1.96 SD, with SD being the observed standard deviation (SD) of the difference between the two measurements per subject.

A paired t test, Wilcoxon signed test, and Mann Whitney test were performed to determine whether the differences between the two group means were statistically significant.

Kaplan-Meier survival curves were used to compare survival between responders and non-responders, whilst the non-parametric Log rank tests with significant p value <0.05 was applied to find the significance of the results. The hazard ratio was also defined by using the Cox proportional regression model to identify any significant differences between the two groups[154].
Chapter 4: Experiments

Aims and Hypothesis

Hypothesis of this thesis was that assessment of global liver blood flow by a microbubble ultrasound derived hepatic index known as CE-HPI can predict an early response to cancer treatment. To test this hypothesis, our plan was

1. To conduct the invitro/phantom study to find out whether changes in liver blood flow parameters are reflected through a MICROBUBBLE ULTRASOUND derived hepatic index (i.e. CE-HPI).

2. To determine the range of CE-HPI in healthy as well as patients with liver tumours without any treatment.

3. To assess variability of CE-HPI with respect to inter-operator, inter-scan, fasting versus non-fasting factors and quantification on Q-lab software.

4. To compare this CE-HPI value in patients with treatment at baseline and 2 weeks with the response assessment (i.e. RECIST) at 3 months to find out whether this can be helpful in predicting the response.

5. Finally, to assess the feasibility of CE-HPI to ascertain changes in liver haemodynamics at various phases in patients receiving SIRT.
Experiment 4.1 Title: Quantitative analysis of contrast bolus kinetics and microbubble ultrasound derived blood flow parameters to reflect global liver blood flow in phantom (invitro)

Background
Doppler studies by various researchers have demonstrated the potential for quantitative assessment of altered hepatic haemodynamics (i.e. hyper-arterialisation) as a result of development of liver metastases. They found an increased hepatic arterial and reduced portal venous flow component to total liver blood in terms of volumetric flow (i.e. volume of fluid passing through surface per unit time). Unfortunately, due to the requirement of higher operating skills, these studies were not repeated in other centres. With the introduction of microbubbles as contrast agents, there is potential to obtain time intensity curves from regions of interest over hepatic artery and portal vein and subsequently derive different liver blood flow parameters, such as peak intensity (PI), rise time (RT), wash in slope (WIS), which give an indirect estimation of the total hepatic flow in terms of volumetric flow. However, there is a lack of experimental data to establish the relationship between microbubble ultrasound derived blood flow parameters and characteristics of liver blood flow, such as volumetric flow rate and velocity.

Objective
This experiment was conducted to assess the quantitative analysis of microbubble ultrasound derived blood flow parameters to reflect global liver blood flow changes in phantom (invitro).
Protocol
The study was conducted, from May 2010 to June 2010. The set up (figure 22) consisted of two tubes of 2 and 5 mm diameter, simulating the hepatic artery and portal vein respectively in the liver. These were immersed in degassed and de-ionized water at ambient temperature 25 °C at a depth of 2.5 cm - a typical depth of imaging as in a clinical scenario. The tubes were connected alternatively to the input and output of the flow phantom, which consisted of a 1-Litre reservoir as input and a tube running through a peristaltic pump connected to the output reservoir.

The ultrasound machine was a Philips IU22 scanner (settings as given in chapter 3, page 75) with L12-5 linear transducer, positioned in the transverse plane (perpendicular to the direction of the tubes). SonoVue was used as the contrast agent and was delivered as 0.05 and 0.1 ml boluses. One minute image loops were acquired twice, for each acquisition, and then transferred to a laptop with QLAB quantification software for offline analysis. The regions of interest were drawn over the cross sections of the two tubes (2 mm and 5 mm tube) to derive two separate time intensity curves.

Methods
SonoVue preparation, method of injection, imaging technique, image transfer, quantification, image analysis and statistical analysis of blood flow parameters, response assessment are described in methods, chapter 3, and page 66-86.
Figure 22. Indicator dilution setup,

It consisted of

1. 1 litre reservoir of degassed and de-ionized water at temperature 25 °C
2. Syringe filled with ultrasound contrast agent-SonoVue microbubbles
3. IU22 scanner
4. C-5-1 curvilinear transducer
5. Peristaltic pump- [SP vario PD 5101: Heidolph, Schwabach, Germany] running at constant speed of 20, 30, 40, 50 and 60 rotations per minute, to maintain the constant volume flow.
6. 2 mm tubes with flow velocities of 8.7, 13, 17, 22 and 26 cm/s, and maintaining constant volume flow of 16.5, 24.7, 33, 41.2, and 49.5 ml/min.
7. 5 mm tubes with flow velocities of 8.7, 13, 17, 22 and 26 cm/s, and maintaining flow volumes of 85.5, 128.2, 171, 213.7 and 256.5 ml/min
Results

In both tubes, with increase in volumetric flow, the RT decreased while the PI and WIS increased. The ratios of RT, PI, WIS (2mm tube to 5 mm tube) were also calculated. The RT ratio decreased, while PI and WIS ratios increased with increase in volumetric flow. The RT ratio decreased by 75% (0.05 ml bolus) and 82% (0.1 ml bolus) when the volume flow ratio was increased from 0.06 to 0.58, while the PI and WIS ratios increased by 50% (0.05 ml bolus) and 32% (0.1 ml bolus), and 550% (0.05 ml bolus) and 810% (0.1 ml bolus), respectively (unpaired 2-sided t-test p < 0.05). The relationship between the volumetric flow rate and RT, PI and WIS appeared to be relatively independent of the contrast bolus volume.

Figure 23. a, b, c. Ratios of the RT, PI, and WIS and CE-HPI in the 2 mm tube to those measured in the 5 mm tube as a function of ratio of volumetric flow in the 2 mm tube to 5 mm tube.
The RT ratio decreases, while CE-HPI, PI ratio and WIS slope ratio increase with the increase in volumetric flow ratio. The ratios were calculated to reduce the variability induced by the operator, rate of injection and also the different depths of regions of interest in the liver.

The RT, PI WIS ratios and CE-HPI are taken as the variation in the contrast bolus administration (such as stability of contrast agent, variation in the bolus volume, rate of administration, variability in quantification due to tissue depth and attenuation of ultrasound beams, as well as the variability of the measurement equipment) which affects the HA measurement is “cancelled out” by the same variation in the contrast bolus administration affecting the PV measurement in a similar fashion.

**Discussion**

In this invitro study, the 2 mm and 5 mm tubes mimicked hepatic artery and portal venous blood flow in the liver. There was a decrease in the RT ratio and increase in the PI ratio and WIS ratio due to an increase in volumetric hepatic flow and flow velocity.

There were certain limitations;

First, this experiment did not mimic the clinical scenario of contrast bolus mixing in the heart chambers, which induces an uncertain input function. This uncertainty in the input function is addressed through the disruption replenishment technique (explained in chapter 2.1.6 page 38). Second, the hepatic artery blood flow in real patients is pulsatile and there is a recirculation component after 40 seconds, which may affect the time intensity curves. Third, there is no simulation of fluctuations in the time intensity curves introduced by diaphragmatic motion in real patients. However, these concerns have been addressed in human studies by using respiratory gating technique.

In addition, we used the high frequency linear transducer L-9 in this experiment, while in clinical studies, low frequency curved transducers are used. Microbubbles behave
differently when exposed to sound waves of different frequencies. However, this was unlikely to affect our study, as this study did not entail head to head comparisons with clinical studies or experiments.

Summary

The RT ratio (indicative of rate of filling of microbubbles, i.e. velocity of blood flow) decreased with increased volumetric flow, indicating increased velocity of blood flow in the hepatic artery, compared to the portal vein. The PI ratio (i.e. vascular volume) increased with increased volumetric flow, and the WIS ratio (i.e. velocity of blood flow) also increased with increased volumetric flow. This suggests that increase in arterial blood flow can be assessed by microbubble ultrasound.
Experiment 4.2 Title: Quantitative assessment of microbubble ultrasound derived blood flow parameters in healthy volunteers as well as in patients with primary or metastatic liver tumours

Background

Doppler perfusion studies and the contrast enhanced Doppler perfusion index by Ramnarine et al. (2002) suggested the reversal of normal portal liver haemodynamics (i.e. hyper-arterialisation) in tumours through ultrasound derived blood flow parameters [134]. To date there are no experimental data to indicate the range of microbubble ultrasound derived blood flow parameters in healthy volunteers and patients with liver tumours. Dynamic contrast enhanced ultrasound using microbubbles as a contrast agent was performed to quantify different liver blood flow parameters in controls (without any malignant liver lesions) and patients with confirmed liver malignancies (both primary and metastatic). The aim was to identify significant parameters which could be later on used for monitoring response to therapy, to assess the potential for early response assessment for treatment for liver tumours.

Methods

SonoVue preparation, method of injection, imaging technique, image transfer, quantification, image analysis and statistical analysis of blood flow parameters as described in methods chapter 3, page 66-86.

Patient recruitment

In this experiment conducted from June 2010 to July 2010. Twenty controls (healthy volunteers, scanned by EL, with 20 years’ experience of ultrasound) and 36 patients (without any treatment, scanned by AM, with experience of 100 ultrasound scans) had a single baseline contrast ultrasound scan with 2 ml SonoVue microbubbles.
Controls (healthy volunteers) and patients

The healthy controls (n=20) were being investigated for suspicious liver lesions (without any cancer history) in the ultrasound department of Hammersmith Hospital. The following diagnosis was confirmed after contrast ultrasound scans: Patients with cysts n= 3 fatty changes n=3  haemangiomas n=3  normal n= 11

Patients (excluding healthy controls) included in these experiments had the following demographics and characteristics (table 6).

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>n</th>
</tr>
</thead>
<tbody>
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</table>

Table 6. Patient characteristics and demographics at baseline
Results

Peak intensity ratio (PI HA†/ PI PV‡)

<table>
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<th>PI ratio</th>
<th>Number of patients</th>
<th>Mean (SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>20</td>
<td>1.15 (0.56)</td>
<td>0.0008*</td>
</tr>
<tr>
<td>Patients</td>
<td>36</td>
<td>3.24 (3.07)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7. Peak Intensity ratio of patients and healthy controls.** The PI (i.e. vascular volume) was the only parameter found to be significantly different (p<0.05) at baseline between controls and patients. The increased PI ratio indicates increased arterial blood flow in patients with liver tumours as compared to healthy controls. *statistically significant (p value <0.05) † Peak intensity of hepatic artery ‡Peak intensity of portal vein

Contrast Enhanced Hepatic Perfusion Index (CE-HPI) Patients vs. controls

<table>
<thead>
<tr>
<th>CE-HPI</th>
<th>Number of patients</th>
<th>Mean (SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>20</td>
<td>3.5 (3.3)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Patients</td>
<td>36</td>
<td>32.0 (55.5)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 8. CE-HPI of patients and healthy controls.** CE-HPI was found to be significantly higher (p=0.03) in patients, again indicating arterialisation and consequently increased arterial volumetric blood flow in patients with liver tumours.*statistically significant (p<0.05)

Wash in slope ratio (WIS HA/WIS PV) and Rise time ratio (RT HA/RT PV)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls n=20</th>
<th>Patients n=36</th>
<th>Mann Whitney test p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIS ratio</td>
<td>2.98 (1.88)</td>
<td>7.3 (11.2)</td>
<td>1</td>
</tr>
<tr>
<td>RT ratio</td>
<td>0.54 (0.29)</td>
<td>5.57 (16.05)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Table 9. WIS ratio and rise time ratio of patients and healthy controls.** WIS ratio, i.e. the velocity of blood flow ratio from the hepatic artery to portal vein, did not change significantly (p>0.05) between the controls and the patients with liver tumours at baseline. The RT ratio was also not significantly different (p>0.05) between the controls and patients.
Discussion

In the current experiment, microbubble ultrasound was performed to quantify different liver blood flow parameters in healthy controls (without any malignant liver lesions) and patients with confirmed liver malignancies (both primary and metastatic). The aim was to identify significant parameters which could be later on used to assess the potential for early response assessment of treatment for liver tumours.

We found that the PI ratio, which is the ratio of blood volume in hepatic artery to portal vein, and CE-HPI, which is the ratio of hepatic artery volumetric flow rate to portal venous volumetric flow rate, were significantly different (p <0.05) in patients with liver tumours compared to healthy volunteers. Both the PI ratio and CE-HPI indicate an increase in hepatic arterial blood flow and or decrease in portal venous flow in patients with liver tumours. This is consistent with the finding that liver tumours receive the majority of their blood supply from the hepatic artery [155]. The CE-HPI reflects both the blood volume and blood flow in the hepatic artery and portal vein; it is therefore more sensitive to any changes in liver blood flow as compared to individual blood flow parameters such as rise time and wash in slope (blood flow).

The hepatic artery and portal vein ratios achieved statistical significance in differentiating patients from healthy volunteers; while individual parameters such as hepatic artery rise time and peak intensity did not. This could possibly be explained by variations in contrast bolus administration such as bolus volume or rate of administration potentially being cancelled out by the same variations in the portal vein measurements in a similar mode.
This experiment gives rise to the main limitation of this study: potential variability between the two operators in their assessment of liver blood flow parameters. Also the ROC curve indicates the wide overlapping range for CE-HPI values between controls and patients at baseline before starting any therapy. Although the specificity was quite high (95 %) at or above 10 for CE-HPI, sensitivity was quite low (45%), indicating the possibility of missing more than 65 % of patients who could have occult lesions.

Ramnarine et al. (2010) proposed a similar index for assessment of liver blood flow parameters, namely the contrast enhanced Doppler perfusion index (CE-DPI), which was found to be significantly higher in patients with liver metastases (mean value of 0.59) as compared to patients with haemangiomas (mean value of 0.33). They concluded that CE-DPI is a potentially useful method to assess hepatic blood flow parameters as well as focal liver lesions such as haemangiomas and liver tumours[134]. Once it had been determined that the PI ratio and CE-HPI would be more useful for the longitudinal follow up and early response assessment, the next experiment entailed assessing the impact of potential factors affecting the reproducibility and variability of these parameters.

**Summary**
A significant rise in PI ratio and CE-HPI (intensity based parameters) in patients with liver tumours, in accordance with the invitro experiment, indicates an increase in hepatic arterial blood flow and decrease in portal venous flow in patients. Of the entire parameters only PI ratio achieved statistical significance in differentiating healthy volunteers vs. patients, suggesting that arterialisation leads to significant changes in the vascular volume of the hepatic artery whereas changes in velocity of flow (depicted by RT or WIS as time based parameters) are probably more subtle and less useful in differentiating healthy controls.
from patients. The PI ratio and CE-HPI were, hence, found to be more useful for longitudinal follow up and early response assessment. However, in terms of conducting longitudinal follow up and monitoring response, consistency of agreement is the key, so the next phase was to determine the reproducibility of these blood flow parameters.
Experiment 4.3 Title: Assessment of reproducibility of microbubble ultrasound derived liver blood flow parameters in patients with liver tumours

Background
In the previous experiment, peak intensity (PI) ratio and contrast enhanced hepatic perfusion index (CE-HPI) were identified as significantly different blood parameters in patients with liver tumours and also acted as a surrogate of volumetric flow rate in the hepatic artery and portal vein. But the pre-requisite for these surrogate parameters to be translated into clinical studies is the reliability and reproducibility of measurements, which is defined as the repeatability of measurements over a period of time or by different observers[156]. Intraclass correlation coefficients (ICCC), coefficients of variation (COV), and Bland-Altman limits of agreement (explained in chapter 3 page 85) are the most common indicators of reliability and reproducibility[157]. The reproducibility and reliability of these blood flow parameters was determined in patients with liver tumours in this experiment conducted in the Oncology Department at Hammersmith Hospital from June 2010 to August 2010.

Objective
Dynamic contrast enhanced ultrasound using microbubbles was used:

i. To determine inter-scan variability of CE-HPI in patients with histologically confirmed liver neoplasms.

ii. To determine inter-operator variability of CE-HPI in patients.

iii. To determine the postprandial variability of hepatic blood flow parameters including CE-HPI in patients with liver tumours.
Methods

SonoVue preparation, method of injection, imaging technique, image transfer, quantification, image analysis and statistical analysis of blood flow parameters as described in methods chapter 3, page 66-86.

I. Test-retest reliability (repeatability)/ Inter-scan variability

Scans of twelve patients (colorectal cancer n=4, neuroendocrine n=2, hepatocellular carcinoma n=4, gall bladder cancer n= 1, pancreatic cancer n=1) were randomly selected. These patients fasted for 4 hours before undergoing microbubble ultrasound with 2 ml SonoVue, administered twice by the same operator after a 5-10 minute interval.

<table>
<thead>
<tr>
<th>COV</th>
<th>Number of patients</th>
<th>Mean (SD)</th>
<th>COV scan A and Scan B (SD/ Mean) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test scans</td>
<td>12</td>
<td>8.6 (18.5)</td>
<td>45 %</td>
</tr>
<tr>
<td>Retest scans 5 minutes later</td>
<td>12</td>
<td>6.9(14.08)</td>
<td></td>
</tr>
</tbody>
</table>

Table 10. Test re-tests (inter-scan) reliability of CE-HPI, with COV of 45 %

Bland-Altman Chart

Figure 24. Bland-Altman chart for test re-test reliability of CE-HPI showing bias of 0.27 and 95% limits of agreement e from -1.04 to 1.60.

Intraclass correlation coefficient (ICCC) value was 0.90, which indicates high correlation and test-retest reliability of CE-HPI.
ii. Inter-operator variability

Six patients (HCC n=2, CRC n=2, GIST n=2) who had fasted for 4 hours underwent microbubble ultrasound and CE-HPI assessment by two different operators (AM, with experience of 100 scans, and AT, with experience of 50 contrast scans), randomly. The scans were repeated with 2ml SonoVue after an interval of 5-10 minutes for each patient and operator. The quantification analysis on QLAB was performed by AM.

<table>
<thead>
<tr>
<th>COV</th>
<th>CE-HPI</th>
<th>Mean (SD)</th>
<th>COV between AM and AT (SD/Mean) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>5.58 (5.28)</td>
<td></td>
<td>41 %</td>
</tr>
<tr>
<td>AT</td>
<td>5.51(8.52)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11. Inter-operator variability of CE-HPI with COV 41 %

Intraclass Correlation Coefficient (ICCC) was 0.90

Bland-Altman chart

Figure 25. Bland-Altman chart for inter-operator variability of CE-HPI gives bias of only 0.06 and 95% Limits of agreement range from -8.72 to 8.86

Conclusion High ICC and low COV indicate reliability for CE-HPI.
iii. Post prandial effect on CE-HPI

Background

In radiology, the conventional protocol for patients is 4 hours fasting to obtain optimum conditions for ultrasound of the abdomen, such as clear view of the abdomen (avoid gases), to avoid gall bladder contractility and also blood flow changes in the liver due to meals. Several human studies have documented the increased portal venous blood flow, known as postprandial hyperaemia, due to ingestion of food [158]. Postprandial hyperaemia leads to splanchnic vasodilation which then results in an increase in diameter ratio and cross sectional area of the portal vein and significant alteration in the liver haemodynamics [177]. However, four hours fasting has the potential risk of inducing hypoglycemia for diabetic patients. The present study, conducted in the oncology department at Hammersmith Hospital from July 2010 to August 2010, aimed to examine the postprandial variability and effects of meal ingestions on hepatic blood flow parameters, including CE-HPI, in patients with metastatic liver tumours, using microbubble ultrasound.

A group of seven randomly selected (CRC n=3, GB n=1, HCC n=3) patients fasted for at least 4 hours before undergoing microbubble ultrasound and CE-HPI assessment with 2ml SonoVue. These patients were screened for diabetes mellitus and allergies to the contents of chocolate bars. These patients were then given a 56 gram chocolate bar (calories 457 / 100 g, protein 3.4 g/100 g, total fat 17.4 g/100 g, saturated fat 10.3 g/100g, fat trans 0.2 g/100 g, carbohydrate 70.3 g/100 g) after their first scan, followed by another microbubble ultrasound scan after 30-60 min in the same set up, by the same operator. A chocolate bar has conventionally been used as a standard fatty meal, for assessment of fasting studies, gall bladder contractility, and post prandial variability in ultrasound department [158, 159].
The mean, standard deviation (SD) and coefficient of variation (COV) were determined between the fasting and non-fasting states.

Coefficient of Variation (COV)

<table>
<thead>
<tr>
<th>CE-HPI</th>
<th>Number of patients</th>
<th>Mean (SD)</th>
<th>COV between fasting and non-fasting (SD/Mean) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>7</td>
<td>23.71(49.76)</td>
<td>105.06%*</td>
</tr>
<tr>
<td>Non-fasting</td>
<td>7</td>
<td>16.53(21.77)</td>
<td></td>
</tr>
</tbody>
</table>

Table 12. CE-HPI variability in fasting vs. non-fasting state. The COV (>105 %) was high, indicating high variability as a result of postprandial effect. Paired t-test was statistically non-significant (p>0.05).

Two out of 7 patients showed an increase in CE-HPI, while 5 out of 7 had a decrease in CE-HPI, which indicates the possibility of increased portal flow as a result of non-fasting. Two patients did not show the increase in portal effect, which may indicate that such a time frame, is too short to assess inherent variability in a few patients. A trend for post-prandial effect was observed (i.e. increase in portal flow accompanying a reduction in arterial flow) in this set up despite the short interval (30-60 min) between the two scans.

Bland-Altman Chart

Figure 26. Bland-Altman chart for variability of CE-HPI in fasting vs. non-fasting state shows bias of 7.18 and 95 % limits of agreement from -123.0 to 108.6, giving a wide range.
Summary of other blood flow factors (n=7)

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Blood flow parameters</th>
<th>COV for Inter-scan variability between fasting and non-fasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RTHA</td>
<td>0.64</td>
</tr>
<tr>
<td>2</td>
<td>RT PV</td>
<td>0.43</td>
</tr>
<tr>
<td>3</td>
<td>PIHA</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>PIPV</td>
<td>0.71</td>
</tr>
<tr>
<td>5</td>
<td>PI ratio</td>
<td>0.67</td>
</tr>
<tr>
<td>6</td>
<td>CE-HPI</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 13. Blood flow parameter variability in fasting vs. non-fasting state. These blood flow parameters were associated with high variability as a result of postprandial hyperaemia.

Discussion

Very few studies have been conducted in the past to assess the reproducibility of microbubble ultrasound in the liver blood flow.

Tang et al. (2011) studied different sources of variability which affect the quantification analysis. The following factors significantly affected the imaging results and contributed to the variations in their study: ultrasound machine settings, which include output power (mechanical index MI), focal depth, dynamic range, signal gain and transducer frequency, patient characteristics, including body physique differences, physiological interactions of the human body with the microbubbles, tissue motion and attenuation through tissue, microbubble contrast agents, including the type of microbubbles and their stability, preparation and mode of injection (bolus vs. constant infusion), and different dosages[160].

Gauthier et al. (2012), meanwhile, assessed in vitro the impact of different US scanner settings and contrast bolus volume on variability in time intensity curves (TIC) extracted from microbubble US. Along with the contrast bolus volume, they investigated different US
scanner settings such as contrast specific nonlinear pulse sequences, gain, mechanical index (MI), focal zone depth, and acoustic pulse centre frequency, and concluded that these settings should not be changed throughout a longitudinal study. They derived four parameters [peak intensity (PI), area under the curve (AUC), rise time (RT), and mean transit time (MTT)] from the TIC. RT was found to be the most stable parameter, with a coefficient of variation (COV) of 0.7%-6.9 percent. Meanwhile, the COV range was 0.8%-19 % for PI, 12%-71% for MTT and 9.2 %-66 % for AUC (127).

An index similar to CE-HPI, the Doppler perfusion index (DPI), was suggested by Leen et al. (1994) [161]. Subsequently, Oppo et al. (1998) carried out a reproducibility study for DPI parameters (similar to CE-HPI, but using Doppler without contrast injection) and found the interobserver COV and intraobserver COV to be 20% and 16% respectively [162]. Unpublished data from our research group for Doppler studies indicate that HA velocity (in microbubble contrast scans indicated by RT or WIS) is the most stable factor, with a COV of 6 %, while 14 % was reported for DPI. Furthermore, Ramnarine et al. 2002 calculated the COV for CE-DPI, which they found to be 25 % for patients with liver metastases and 31 % for patients with haemangiomas [134].

In a tumour perfusion study by Averkiou et al. (2010), RT or wash in time (WIT) and WIT ratio (WITR = WIT of the tumour / WIT of the normal parenchyma) was found to be the most reproducible and consistent parameter extracted from the TIC of the tumour perfusion [14]. They found the COV 14 % to be for WIT and 9 % for WITR. The PI was more likely to be influenced by machine settings (analogue and digital gain, compression, focus placement and frequency). The other TIC parameters, such as AUC and MTT, required a complete wash-in and wash-out curve lasting more than 2 minutes (table 14) [14].
## Table 14. Summary of reproducibility studies by different research groups.

Meanwhile, in another microbubble ultrasound tumour perfusion study, in patients with HCC, Lassau et al. (2011) found that although the correlation coefficient was high, at 0.80-0.99, the coefficient of variation (COV) between the two operators was also quite high, at >70 %, for tumour perfusion parameters such as peak intensity (PI), time to peak intensity (TPI), wash in slope (WIS) and area under the curve (AUC)[137]. In addition, Williams et al. (2011), who conducted a reproducibility study for ultrasound contrast bolus, reported COVs of 27 % for PI, 29 % for AUC, and 40 % for MTT when tumour perfusion parameters were extracted from the tumour [138]. A further study, by Ng et al. (2012), investigated the reproducibility of DCE-MRI and DCE-CT derived tumour perfusion parameters in an animal...
model of a glioma tumour. The coefficients of variation (COV) for DCE-CT parameters such as blood volume (BV), blood flow (BF), permeability-surface area product (PS) and mean transit time (MTT) were reported as 22%, 25%, 23% and 18% respectively.

For DCE-MRI parameters such as contrast agent reflux rate constant k(ep), endothelial transfer constant K (trans) and extracellular, extravascular space volume fraction v(e) the COVs were 16%, 23% and 20% respectively [164]. In addition, Messiou et al. (2012) assessed the reproducibility of DCE-CT and DCE-MRI scans for assessment of early vascular response in patients with various solid tumours. Patients with pancreatic cancers, colorectal cancers and cholangiocarcinoma were treated with 30 to 45 mg daily cediranib (antiangiogenic therapy). The most reproducible parameters were DCE-MR imaging enhancement fraction (baseline intrapatient coefficient of variation COV= 8.6%), volume transfer constant (COV = 13.9%), and integrated area under the contrast uptake curve at 60 seconds (COV = 15.5%). On DCE-CT, the COV was > 30% for blood plasma volume and 16.0% for positive enhancement integral, whilst DCE-MRI was found to have better reproducibility and therefore to be the modality of choice in clinical trials [165].

To avoid such variability, in the current study the machine settings were kept the same and also the same amount of microbubbles SonoVue 2 ml boluses was used throughout. In the variability and reliability experiments (interscan analysis, interobserver, fasting vs. non fasting) for the global liver blood flow parameters, the COVs were 20-25% for RT, PI, WIS and CE-HPI, which were comparable figures to those reported in other reproducibility studies or with TIC parameters extracted from tumour perfusion. This means any changes in values for these parameters greater than the COV are due to intervention rather than inherent variability.
This pilot study was performed at a single centre, with a limited population of patients. Although these results do indicate the reproducibility and reliability of CE-HPI, these reproducibility studies need to be further verified through being performed in different centres, by different operators, on a larger scale.

**Post prandial variability**

Past studies investigating hepatic hemodynamic changes in patients with cirrhosis have revealed prandial hyperaemia to be at a maximum 30 minutes after ingestion of food, which meant that hyperaemic changes (increased portal venous flow and reduced arterial flow) were found to be consistent in all subjects regardless of whether they were healthy volunteers or patients with liver cirrhosis\[166\]. The effect of meal composition on postprandial hyperaemia has also been investigated. Carbohydrate ingestion has been found to result in increased splanchnic and portal venous blood flow\[82, 167, 168\]; however, other studies have indicated that only proteins induce increased total liver blood flow or splanchnic blood flow \[123, 169\]. Moreover, a few studies have failed to find any correlation between the extent of haemodynamic changes and meal composition, with the only difference being the timing of response \[170\].

Nonetheless, Kawasaki et al. (1990) supported the theory that with food ingestion there is an increase in the cross sectional diameter of the portal vein, resulting in increased portal flow, and an increase in the hepatic arterial resistance index, indicating vasoconstriction of the intrahepatic arterioles \[171\]. In another study, after giving standard meals to 30 healthy volunteers and 12 liver allograft recipients (with cirrhosis), Doppler ultrasound was performed to assess blood flow in the hepatic arteries, superior mesenteric artery and the portal vein. The resistive index (measure of resistance to blood flow calculated as systolic
pressure – diastolic pressure / systolic pressure) was evaluated to reflect the changes in hepatic arterial resistance. Ingestion of meals resulted in increased portal venous flow and reduced superior mesenteric artery resistance, while there was an accompanying increase in hepatic arterial resistance, reflecting constriction of the hepatic arteries, in all subjects. These postprandial haemodynamic changes were more conspicuous in allograft recipient patients and were at a peak 30 minutes after the ingestion of meals [172].

In the current study, the trend towards postprandial haemodynamic changes in terms of hepatic blood flow parameters was observed (albeit statistically non-significant due to small sample size). The peak intensity (PI) of the portal vein, which indicates the blood volume in the portal vein, was increased while the hepatic artery blood volume either remained constant or decreased about 30 minutes after the ingestion of 56 grams of a chocolate bar containing fats, proteins and carbohydrates. The finding was consistent with previous studies indicating that maximum postprandial hyperaemia is obtained 30-60 minutes after food ingestion. In order to achieve consistent results in this thesis, 30-60 minutes of fasting was insufficient; therefore the conventional protocol of 4 hours fasting was adopted. The limitations of this study were the small sample size and adoption of a time interval of 30-60 minutes. Hence, a randomized study of a large sample of patients and performance of microbubble ultrasound examination at time intervals of 1 hour, 2 hours and 4 hours will potentially give more insight into post-prandial changes in hepatic haemodynamics.
Summary

The interscan and inter-operator variability of the parameters of PI ratio and CE-HPI is 15% to 25%. In the longitudinal follow up, the meaningful values have to be above this level of variability. Immediate post prandial variability is very high, so the conventional fasting ultrasound protocol of 4 hours used in ultrasound departments was adopted for all studies. Patients were allowed to drink water and diabetics were prewarned to ensure they ate their regular breakfast and also educated about hypoglycemia symptoms such as dizziness and palpitations. They were also instructed to take necessary precautions and keep sugar tablets with them in case these symptoms arose.
Experiment 4.4 Title: Reproducibility of quantitative assessment of microbubble ultrasound derived liver blood flow parameters in patients with liver tumours

Background

The QLAB software (Philips Health care, Andover, MA) is specialized quantification software to view and quantify microbubbles contrast dynamics (time intensity curves) in the hepatic artery (HA) and portal vein (PV) on a Philips ultrasound machine. It can be used for the on-line and off-line review and quantification of ultrasound imaging studies. This software allows for manual region of interest (such as hepatic artery, portal vein, normal liver parenchyma, liver tumours) selection, measurement of the selected ROI area and provides linear (logarithmic compression removed) data for the time intensity curves [173].

In QLAB Quantification software, there is the possibility of intra-reader variability or variability when quantification is performed by different readers. Hence, this experiment, conducted in Hammersmith Hospital oncology department from June 2010 to August 2010, was aimed to assess the reproducibility of quantitative assessment of alterations in liver haemodynamics with microbubble ultrasound derived blood flow parameters.

Objective

The objective was to determine inter-reader and interscan (single reader) variability of quantification analysis at different time intervals, 1 week apart (patients without any treatment).

Methods

The SonoVue contrast preparation, method of injection, machine settings, imaging technique, quantification on QLAB software are as given in the methods (chapter 3, page 66-86).
Protocol

Fifteen patients (age 28-76 years) with biopsy proven colorectal cancer and liver metastases underwent microbubble contrast ultrasounds prior to treatment.

For quantification analysis with respiratory gating protocol, inter reader agreement was assessed by reader 1 (AM, with experience of 100 scans) and reader 2 (TG, with experience of 200 scans). For inter-reader analysis, the readers were blinded to each other and also to the clinical information on the patients. Both readers followed the same quantification protocol as given in the methods (page 66-86). A single reader (reader 1 AM) assessed the inter scan agreement by analysing all scan studies twice (with a 2-month interval to reduce recall bias).

Results

A. Mean (SD) and COV

The rise time, peak intensity and WIS ratios in patients with colorectal cancer and liver metastases are summarised in table 15 (Mean (SD) and the coefficients of variation are shown in table 16. The rise time ratio (RT HA/RT PV) for the blood flow parameters demonstrated intra-reader and inter-reader agreement, with COV ranging from 12% (intra-reader) to 24% (inter-reader). The peak intensity ratio (PI HA/PI PV) also showed intra-reader and inter reader agreement, with COV ranging from 8% (intra-reader) to 15% (inter-reader).

The level of agreement for the WIS ratio (WIS HA/WIS PV) was comparable to other blood flow parameters, with a COV ranging from 12% (intra-reader and inter-scan) to 15% (inter-reader agreement).
Table 15. PI ratio, and WIS ratio values mean (SD) for patients with colorectal liver metastases for scans 1 and 2 (ten minutes apart) and performed by both readers (AM and TG).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reader 1, scan 1</th>
<th>Reader 2, scan 1</th>
<th>Reader 1, scan 2</th>
<th>Reader 2 scan 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIS ratio</td>
<td>17.7 (12.8)</td>
<td>17.4 (9.1)</td>
<td>16.3 (8.1)</td>
<td>16.4 (7.0)</td>
</tr>
<tr>
<td>PI ratio</td>
<td>3.5 (1.4)</td>
<td>3.4 (1.0)</td>
<td>3.8 (2.5)</td>
<td>3.7 (2.3)</td>
</tr>
<tr>
<td>CE-HPI</td>
<td>61.9 (4.2)</td>
<td>59.6 (9.1)</td>
<td>61.94 (20)</td>
<td>60.68 (16)</td>
</tr>
</tbody>
</table>

Table 16. Coefficients of variation (SD/Mean) for inter-reader, inter scan variability. These are in the range of 15% to 18%.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inter-reader COV</th>
<th>Interscan COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI ratio</td>
<td>10%</td>
<td>15%</td>
</tr>
<tr>
<td>CE-HPI</td>
<td>15%</td>
<td>18%</td>
</tr>
</tbody>
</table>

B. Intraclass correlation coefficient

Intraclass correlation coefficient as a parameter indicates both qualitative and quantitative aspects of inter-reader, intra-reader and inters scan agreement (shown in Table 17).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inter-reader</th>
<th>Intra scan</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI ratio</td>
<td>0.948</td>
<td>0.978</td>
</tr>
<tr>
<td>WIS ratio</td>
<td>0.909</td>
<td>0.968</td>
</tr>
<tr>
<td>CE-HPI</td>
<td>0.86</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 17. Intraclass correlation coefficients for inter-reader and inter-scan variability. These are in the range of 0.8-0.9, which indicates high reproducibility.

C. Bland-Altman Plots

Bland-Altman plots of PI ratio and WIS ratio have been shown in figure 27, indicating a level of agreement with no more than 1 of 20 points falling outside the 95% reference range (Mean (2SD).
A. Bland-Altman plot for PI ratio-inter reader agreement

B. Bland-Altman plot for PI ratio-inter scan agreement

C. Bland-Altman plot for CE-HPI-inter reader agreement
D. Bland-Altman plot for CE-HPI interscan agreement

**Figure 27.** A, B, C, D Bland-Altman plots for PI ratio inter-reader and inter-scan and CE-HPI inter-reader and inter-scan respectively. Bias is -1.7 and 95 % of limits of agreement range from -6.5 to 3.0 for interscan and inter-reader PI ratio. Bias is 1.2 and 95 % of limits of agreement range from -12.2 to 14.8 for CE-HPI.

**Discussion**

Gauthier et al. (2011), in an in-vitro microbubble ultrasound study, found rise time (RT) to be the most stable parameter, with a coefficient of variation (COV) of 0.7-6.9 percent, whilst the COV range was 0.8%-19 % for PI, 12%-71% for MTT and 9.2 %-66 % for AUC [163]. In another variability study, Gauthier et al. (2012) investigated intra-operator variability for quantification, derived from the TIC of the tumour, following bolus injection of SonoVue, both in vitro and in vivo. They found that intra-operator variability ranged from 2%-24 % for different volumes of SonoVue (0.02-0.4 ml) and concluded that the AUWO curve and AUC are the most stable parameters in terms of variability [174].

In the current study reproducibility for quantification of blood flow parameters was found to be high, with COVs of 10-15 % for the TIC parameters (RT, PI and WIS) derived from the hepatic artery (HA) and COVs of 5-12 % for the same parameters derived from the portal
vein (PV). The COV was 8-15 % for the ratio of HA/PV parameters and 18-25 % for CE-HPI, [174]. When conducting a longitudinal follow-up and early response assessment with these blood flow parameters, any meaningful change has to be in excess of this range (15-25%). Hence, PI ratios and CE-HPI with percentage reductions greater than 25 % were considered for categorisation of patients as responders, whilst non responders were identified as those where the change was < 25 % or where there was an increase.

Summary

Quantitative assessment of microbubble ultrasound derived liver blood flow parameters with QLAB software is reproducible and consistent with variability in the range of 15 % to 18 %, if measurements in the hepatic artery are normalised by those in the main portal vein, especially the peak Intensity ratio and CE-HPI. After determination of reproducibility of these parameters, the next phase was to assess the altered liver haemodynamics and evolution of liver blood flow parameters over time to assess early response to treatments including chemotherapy and antiangiogenic therapy.
Experiment 4.5 Title: Quantitative assessment of Microbubble ultrasound derived liver blood flow parameters for early response assessment in patients with liver tumours treated with systemic cytotoxic chemotherapy and anti-angiogenic therapy

Background

Preliminary tumour perfusion studies on microbubble ultrasound by various researchers have indicated its potential to predict early treatment response in patients receiving antiangiogenic therapy and combination chemotherapy [14, 136, 137]. However, perfusion of a single index liver tumour was assessed in these studies to monitor response to therapy, whereas there are multiple occult micro-metastases in the liver, and these may behave differently in terms of response in quantification of tumour perfusion. Response assessment based on total liver blood flow over the entire liver tumour therefore has an advantage over tumour perfusion studies conducted in the past.

In the current experiment, conducted in the oncology department at Hammersmith Hospital from Sep 2010 to June 2012, emphasis was placed on global liver blood flow parameters rather than perfusion of individual tumours.

Objective

The objective was to determine the evolution of flow parameters from the time intensity curve over the treatment period (baseline and 2 weeks after treatment) and to determine whether it can predict response to individual treatment regimens, including chemotherapeutic and biological agents. The secondary objective was to determine correlation between these parameters, progression free survival (PFS), and overall survival (OS) and to prospectively determine the utility of DCEUS with quantification as a prognostic tool for the patients.
Methods
The SonoVue contrast preparation, method of injection, machine settings, imaging technique, quantification on QLAB software, response assessment, statistical analysis are as given in the methods (chapter 3, pages 66-86). Chemotherapy regimens are described in Appendix 5.

Patient recruitment

Figure 28. Patient Recruitment summary. PD=progressive disease PR=partial response, SD=stable disease. * Scan quality was audited by MA, who has 10 years’ experience of quantification of images. Two out of 5 scans were declared inadequate due to obesity, and 3 due to inadequate quality of images.
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with baseline 2 weeks scans, contrast scans and RECIST</td>
<td>65</td>
</tr>
<tr>
<td>Mean Age (range)</td>
<td>65 (30-83)</td>
</tr>
<tr>
<td>Patients with PS 2</td>
<td>22</td>
</tr>
<tr>
<td>Patients with PS 1</td>
<td>43</td>
</tr>
<tr>
<td>Males</td>
<td>48</td>
</tr>
<tr>
<td>Females</td>
<td>17</td>
</tr>
<tr>
<td>Patients with cholangiocarcinomas</td>
<td>6</td>
</tr>
<tr>
<td>Patients with pancreatic cancer</td>
<td>6</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>25</td>
</tr>
<tr>
<td>HCC</td>
<td>12</td>
</tr>
<tr>
<td>GIST</td>
<td>4</td>
</tr>
<tr>
<td>NETs</td>
<td>10</td>
</tr>
<tr>
<td>CUP</td>
<td>2</td>
</tr>
<tr>
<td>Patients with 1st line anti-cancer treatments</td>
<td>31</td>
</tr>
<tr>
<td>Patients with 2nd line anticancer treatments</td>
<td>24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Median PFS (Days)</th>
<th>Median OS (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with SD(n=37)</td>
<td>230</td>
<td>335</td>
</tr>
<tr>
<td>Patients with PR(n=9)</td>
<td>259</td>
<td>704</td>
</tr>
<tr>
<td>Patients with PD(n=19)</td>
<td>70</td>
<td>114</td>
</tr>
</tbody>
</table>

Table 18. Summary of patient characteristics PS: Performance Status (Appendix 1). Patients with SD and PR were categorised as responders and PD as non-responders according to RECIST at 3 months. PFS progression free survival OS overall survival
Results

Peak Intensity Ratio (PI HA† / PIPV‡)

<table>
<thead>
<tr>
<th>PI ratio</th>
<th>Mean (SD)</th>
<th>2 weeks</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders (N=46)</td>
<td>5.82 (9.16)</td>
<td>2.99 (5.28)</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Non-Responders (N=19)</td>
<td>3.24 (2.90)</td>
<td>11.48 (18.90)</td>
<td>0.03*</td>
</tr>
</tbody>
</table>

Table 19. PI ratio: comparison between responders and non-responders *statistically significant (Wilcoxon rank sum test p value < 0.05)† PIHA Peak intensity of hepatic artery , ‡PIPV peak intensity of portal vein.

Figure 29. PI ratio: Comparison between responders and non-responders. Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. The mean PI ratio was reduced (by 49%) in responders (according to RECIST) while in non-responders (according to RECIST) it was increased (by 254%). Increased PI ratio indicates the arterialisation leading to progressive disease in non-responders, which was shown as increased vascular volume of the hepatic artery as compared to the portal vein.
Contrast enhanced hepatic perfusion index (CE-HPI)

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>2 weeks</td>
<td>(p value)</td>
</tr>
<tr>
<td>Responders (N=46)</td>
<td>278 (1281)</td>
<td>58.4(181.80)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Non-Responders (N=19)</td>
<td>60.30 (105.7)</td>
<td>649.50 (1859)</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

Table 20. CE-HPI: comparison between responders and non-responders *statistically significant (Wilcoxon rank sum test p value < 0.05)

Figure 30. CE-HPI: Comparison between responders and non-responders. Semi-logarithmic (log 10) scale is applied on y-axis to separate overlapped readings. The mean CE-HPI was reduced (by 99%) in responders while in non-responders it was increased (by 977%). The CE-HPI showing blood volumetric flow ratio in the hepatic artery to the portal vein had decreased in responders whilst in non-responders it had increased.

Other blood flow parameters

The PI of HA (vascular volume) was significantly reduced (p=0.018) in responders, indicating reduced arterialisation leading to a decrease in vascular volume of the hepatic artery.

Meanwhile, in non-responders there was a non-significant decrease (p=0.368). This might indicate a trend towards an increased arterial blood flow or no de-arterialisation and subsequently further progression in non-responders at 2 weeks. Peak intensity of the portal vein (PI PV) had non-significantly increased (p=0.23) in responders, possibly showing the trend towards increased portal venous flow as a result of liver homeostasis. Meanwhile, in
non-responders there was a significant reduction in PIPV (p=0.023), which means there was less portal flow and more hepatic arterial flow in these patients. Other hepatic blood flow parameters, such as RTHA and RTPV, did not change significantly in either responders or non-responders.

**Progression free survival (PFS) and Overall survival (OS)**

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-Responders</th>
<th>Log-rank Test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFS (days)</strong></td>
<td>245</td>
<td>119</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>OS (days)</strong></td>
<td>373</td>
<td>239</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Table 21: PFS and OS for responders and non-responders (according to CE-HPI ≥ 25% reduction) and non-responders (according to CE-HPI reduction <25 % or increase).* Based on mean CE-HPI, PFS was 245 days in responders and 119 days in non-responders (hazard ratio 0.48 with 95 % confidence interval (0.2-0.8)). There was significance difference between responders and non-responders, when log-rank test was applied (p=0.003). The OS was 373 days in responders and 239 days in non-responders (hazard Ratio 0.48 with 95 % confidence interval (0.2-0.8)). There was significance difference between responders and non-responders on basis of OS, when log-rank test was applied (p=0.014).

**Summary**

In summary, the findings in the current study indicated the ability of microbubble ultrasound derived blood flow parameters PI ratio and CE-HPI at 2 weeks after the beginning of the treatments to correctly predict the response as characterized by RECIST at 3 months. PFS and OS are the most important measures of efficacy. Correlation was also identified between CE-HPI and PFS and OS. This shows the potential of quantification analysis of total hepatic blood flow with microbubble ultrasound derived blood flow parameters for early response assessment in patients with liver tumours receiving heterogeneous treatments.
Subgroup analysis

4.5.1: Antiangiogenic biological agents and chemotherapy combinations

Thirty patients were analysed and assessed for response in this study. Eight patients with HCC had Sorafenib, 2 patients with HCC had axitinib, 2 HCC patients received brivanib, one patient with GIST had imatinib, and 2 GIST patients had sunitinib as a 2nd line therapy. Four patients with NETs and liver metastases had sunitinib and 1 had bevacizumab, 8 patients with CRC and liver metastases had bevacizumab (Avastin) in combination with chemotherapy, and 1 CRC patient with CRC had cetuximab monotherapy.

Figure 31. Patient recruitment summary.

SD = stable disease, PD= progressive disease, PR=partial response. RECIST= response evaluation criteria in solid tumours. Patients with SD and PR were categorised as responders and PD as non-responders according to RECIST at 3 months. * Scan quality was audited by MA, who has 10 years’ experience of quantification of images.
<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients analysed and assessed for response</td>
<td>30</td>
</tr>
<tr>
<td>Males</td>
<td>21</td>
</tr>
<tr>
<td>Females</td>
<td>9</td>
</tr>
<tr>
<td>Mean age(years)</td>
<td>60</td>
</tr>
<tr>
<td>Age range(years)</td>
<td>41-80</td>
</tr>
<tr>
<td>Patients with PS 1</td>
<td>18</td>
</tr>
<tr>
<td>Patients with PS 2</td>
<td>12</td>
</tr>
<tr>
<td>Hepatocellular carcinoma (HCC)</td>
<td>12</td>
</tr>
<tr>
<td>Gastrointestinal stromal tumour (GIST)</td>
<td>4</td>
</tr>
<tr>
<td>Neuroendocrine tumours (NETs)</td>
<td>5</td>
</tr>
<tr>
<td>Colorectal cancer (CRC)</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 22. Summary of patient demographics and characteristics

Results

Peak Intensity Ratio (PI Ratio)

<table>
<thead>
<tr>
<th>PI ratio</th>
<th>Mean (SD) Baseline</th>
<th>Mean (SD) 2 weeks</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders (N=22)</td>
<td>7.01 (12.3)</td>
<td>2.85 (5.36)</td>
<td>0.0023*</td>
</tr>
<tr>
<td>Non-Responders (N=8)</td>
<td>3.19 (2.36)</td>
<td>15.03 (28.31)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Table 23. PI ratio at 2 weeks: compared between responders and non-responders  *statistically significant (Wilcoxon rank sum test p value < 0.05). The baseline comparison between two groups was statistically non-significant (Wilcoxon rank sum test p value > 0.05).
Figure 32. PI ratio at 2 weeks: comparison between responders and non-responders. Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. There was significant reduction (59%) in mean PI ratio (p=0.0023) in responders. As PI ratio indicates vascular volume of the vessel, this shows that there was a reduction in vascular volume of the hepatic artery due to de-arterialisation and a decrease in new arterial recruitment (neo-angiogenesis) in responders.

The PI ratio changes in responders correspond to the anti-vascular effects of antiangiogenic drugs as seen in preclinical models [94]. Meanwhile, in non-responders the PI ratio did not change significantly, indicating that the liver tumours in these patients did not de-arterialise or showed any response to anti-angiogenic therapy.

**Contrast Enhanced Hepatic Perfusion Index (CE-HPI)**

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>2 weeks</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CE-HPI</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>2 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>Responders (N=22)</td>
<td>482.3 (1869)</td>
<td>51.63 (179.70)</td>
<td>0.0039*</td>
</tr>
<tr>
<td>Non-Responders (N=8)</td>
<td>50.88 (68.01)</td>
<td>995 (2756.0)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Table 24. CE-HPI at 2 weeks: Comparison between responders and non-responders *statistically significant (Wilcoxon rank sum test p value < 0.05). The baseline comparison between two groups was statistically non-significant (Wilcoxon rank sum test p value > 0.05).
Figure 33. CE-HPI at 2 weeks: Comparison between responders and non-responders.

Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. There was significant reduction (89%) in mean CE-HPI in responders. The CE-HPI indicates the blood volumetric flow ratio from the hepatic artery to the portal vein. There was a significant reduction (p=0.0039) in CE-HPI at 2 weeks in responders, which shows that blood volumetric flow was reduced in the hepatic artery due to decreased arterialisation and antivascular effect of antiangiogenic therapy.

The CE-HPI had increased in non-responders (albeit non-significantly), which might indicate a trend towards progressive arterialisation leading to increased blood volumetric flow in hepatic artery as compared to portal venous flow, or no de-arterialisation in non-responders.

**Other hepatic blood flow parameters**

Other hepatic blood flow parameters, including RTHA, RTPV and PIHA, did not change significantly between responders and non-responders possibly due to subtle changes in time based parameters and variation in individual intensity parameters.
Progression free survival (PFS) and Overall survival (OS):

PFS and OS (defined in chapter 3 page 84) were determined for these patients.

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-Responders</th>
<th>Log-rank Test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFS (days)</strong></td>
<td>256</td>
<td>156</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>OS (days)</strong></td>
<td>375</td>
<td>235</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 25: PFS and OS for responders and non-responders (according to CE-HPI ≥ 25% reduction) and non-responders (according to CE-HPI reduction <25 % or increase). Based on mean CE-HPI, PFS was 256 days in survivors and 156 days in non-responders. The PFS was significantly different between responders and non-responders, when log-rank test was applied (p=0.02) (hazard ratio of 0.25 and 95 % confidence interval (0.02-0.38)). The OS was 375 days in responders and 235 days in non-responders (hazard ratio 0.25 and 95 % confidence interval (0.02-0.38)). There was no significance difference between responders and non-responders on the basis of OS, when log-rank test was applied (p=0.09).

Discussion

The original hypothesis was that in patients with liver tumours, aberrant arterialisation reverts to normal with antiangiogenic treatment in responders, while either it continues to increase or there is no de-arterialisation effect in non-responders. In this experiment (4.5.1) PI ratios and CE-HPI were reduced in responders whereas the opposite effect was identified in non-responders. The increase in these blood flow parameters indicates an increase in arterialisation with treatment in non-responders, leading to increased calibre and consequently volumetric blood flow in the hepatic artery, whilst patients in the responder group exhibited de-arterialisation at 2 weeks. The other significant finding was that patients in the responder group had significantly high vascularity at baseline, while non-responders had low vascularity. Low vascularity means reduced delivery of chemotherapeutic drug combinations and less response at the end. One implication of this study could be that tumours with greater vascularity could be more likely to respond to antiangiogenic drug
combinations, whereas avascular tumours may not have a high response rate. Ablative techniques such as radiofrequency ablation, ethanol ablation, microwave ablation and cryotherapy techniques might be more suitable for these tumours if they fulfill the relevant criteria of number and size of lesions [175].

Another inference which could be drawn from this study is that antiangiogenic therapy not only affects the microvasculature of the tumour but also affects the major vessels in the liver. Microbubble ultrasound derived liver blood flow parameters indicate that the vessels’ calibre also increases or decreases and consequently volumetric blood flow at hepatic artery and portal venous level changes depending on the response at tumour level. Bonnin et al. (2007) described Doppler technique measurement of hepatic blood flow as a useful indicator of tumour progression in veterinary studies [176]. Meanwhile, Doppler ultrasound has been used in many studies to assist in identification and characterization of liver metastases by measuring blood flow parameters from the hepatic artery and portal vein [4, 177].

In patients treated with antiangiogenic therapy alone or in combination with cytotoxic chemotherapy, diffuse narrowing of the hepatic artery has been observed in hepatic angiography in veterinary models. Wang et al. (2012)[178] demonstrated in a CT perfusion and hepatic angiography study that antiangiogenic therapy leads not only to a reduction in the number of new vessels, but also reduces the size of the pre-existing main vessels. In addition, the tumour vessels became clearer and morphologically more similar to normal tissue vessels, which was consistent with the phenomenon of normalisation. Jain et al. (2005) also described normalisation of the tumour arterial tree during anti-angiogenic therapy[91].
As discussed in chapter 2.4 pages 53-59, reduced hepatic arterial blood flow is associated with an increase in portal flow to maintain liver homeostasis. Therefore, with a reduction in hepatic arterial blood flow resulting from improved control of arteriolar blood flow, there is an increase in portal venous flow due to the reciprocal relationship between portal venous flow and the hepatic arterial flow to maintain haemostasis. Antiangiogenic therapy alone or in combination with cytotoxic chemotherapy, therefore, may have a direct effect at the level of the main hepatic artery through helping to reduce arterial flow to both the liver and tumours.

Willett et al. (2009), in a contrast ultrasound study, demonstrated that vascular volume, interstitial fluid pressure and microvascular density were reduced within 24 hours of bevacizumab infusion in patients with rectal cancer. The number of viable, circulating endothelial and progenitor cells was also decreased, with an increase in the fraction of vessels with pericyte coverage. VEGF inhibition therefore has a direct and quick antivascular effect [95]. These are significantly reduced after treatment with bevacizumab in patients with rectal cancer.

Previously, research groups from France and Canada have reported that microbubble ultrasound might be useful for detection as well as quantification of dynamic early changes in tumour vascularity and perfusion antiangiogenic therapy in different tumour groups, including gastrointestinal stromal tumours (GIST), renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC) [136] [138].

In a phase II study of single-agent bevacizumab treatment, Lassau et al. (2011) assessed tumour perfusion in 37 patients with HCC, and found correlation between tumour perfusion and tumour response at 2 months (RECIST), progression-free survival, and overall survival
In another tumour perfusion study, Averkiou et al. (2010) found the hepatic blood flow parameters wash in time (WIT) and WIT ratio (WIT hepatic artery/WIT portal vein) to be the most reproducible parameters, with the lowest COVs (9-14 %). They found that with the help of WITR it was possible to correctly predict an early response (at 2 weeks) in CRC patients with liver metastases receiving antiangiogenic drug therapy (bevacizumab) [14].

Unlike the earlier tumour perfusion studies, this study placed emphasis on global liver blood flow parameters rather than perfusion of individual tumours, the reason being that with the help of liver blood flow parameters, response over the entire liver tumour can be assessed. There are multiple occult micro-metastases in the liver, and these may behave differently in terms of response in quantification of tumour perfusion. This problem can be addressed by performing microbubble scanning using many boluses of contrast agent, whereby earlier contrast injections have an effect on the subsequent injections due to the microbubbles trapped inside the liver sinusoids producing artefacts. However, this will increase the cost and prolong duration of the scanning as at least 10 minutes must elapse between subsequent boluses of microbubbles.

Different categories of biomarkers for investigating the role of antiangiogenic therapy have been described in the literature.

First, Serum VEGF levels at baseline (i.e. before treatment) for bevacizumab (anti-VEGF monoclonal antibody) have been found to be higher in patients with poor prognostic biomarkers, suggesting they can act as prognostic biomarkers. But their role as predictive biomarkers for bevacizumab has not yet been established [72]. VEGF and VEGFR-2 levels were reduced significantly in all patients with metastatic CRC receiving cytotoxic chemotherapy and
bevacizumab, but surprisingly these levels remained lower than at baseline, even at progression [179].

The potential role of bone marrow derived circulating endothelial cells progenitors (BDCEP) and circulating endothelial cells (CECs) as biomarkers has also been investigated and found to be correlated with angiogenesis in cancer patients. In patients with rectal cancer, a study revealed that bevacizumab treatment resulted in reduced frequency of CECs and BDCECP, whilst in GIST patients an increase in CECs was found in those patients who had a better response in comparison to patients with poor prognoses. Further investigation is required to confirm the exact role of VEGF, CECs and BDCECs as surrogate predictive biomarkers [72].

There have also been numerous studies on the role of DCE-MRI as a surrogate imaging biomarker in antiangiogenic therapies [180]. In a study of CRC patients with liver metastases receiving antiangiogenic and cytotoxic chemotherapy, DCE-MRI was used to measure pre-treatment heterogeneity of tumour vessel enhancement, which was found to predict the outcome following treatment. Hence DCE-MRI was found to have potential as a prognostic biomarker [181]. DCE-MRI was used to measure the decrease in vascular permeability (Ktrans), which was also found to be strongly correlated with better response, in patients with hepatocellular carcinoma (HCC) receiving sunitinib[182].

Another potential imaging biomarker for antiangiogenic therapy that has been investigated is dynamic contrast enhanced computed tomography (DCE-CT). In a study of 42 patients with HCC, DCE-CT derived parameters of blood flow, i.e. time to peak intensity (TPI), hepatic perfusion index(HPI), arterial and portal perfusion, were used to compare the tumour region with the surrounding cirrhotic parenchyma. Arterial perfusion and HPI were higher in tumour tissue but TPI was lower, indicating more arterialisation and higher arterial blood
flow in the tumours. These results indicate the value of DCE-CT blood flow parameters in differentiating and characterizing tumour tissue from the surrounding non-cancerous parenchyma [183].

In brief, these studies conclude that Serum VEGF levels have prognostic value but that their role as predictive biomarkers has yet to be explored. BDCECP and CECs have been found to have contradictory roles in terms of being response biomarkers as whilst various studies have found DCE-MRI to be a good predictive and prognostic biomarker, DCE-CT’s role as a biomarker of surrogate response has not yet been demonstrated. In addition, although the literature has cited hypertension as of interest as a biomarker of surrogate response, in this study it was associated with contradictory results.

This has been a single centre, non-comparator single arm study with a small sample size and non-probability sampling technique. In order to further validate the findings, more phase III studies need to be conducted at different centres and also different tumours and techniques need to be analysed with microbubble ultrasound.

Most of the patients screened for this study were having first line treatment. One limitation of our study therefore has been that the small sample size of patients in the non-responder group (on RECIST) affected its capacity for comparison with other studies. Also there was a heterogeneous mix of tumour types and stages, with heterogeneous antiangiogenic agents considered in combination with chemotherapeutic regimes, which adversely affected the power of this study. Furthermore, previous chemotherapeutic treatments are known to induce cirrhosis, which decreases portal venous flow, and possibly artificially raises the arterial blood flow as reflected in the increased PI ratio and CE-HPI. Despite the heterogeneity of treatment types and tumours, the results of this study indicate that global
hepatic blood flow parameters, including the contrast enhanced hepatic perfusion index (CE-HPI) and PI ratio, could be useful for investigating early response in different treatment groups and regimens and may capture a generic response to treatment.

If validated in large cohort studies, microbubble ultrasound derived global blood flow parameters could be a potential tool for predicting an early response and be used as surrogate imaging response biomarkers.

**Summary**

For the subgroup of patients who received either antiangiogenic therapy alone or in combination with chemotherapies, results indicate the ability of microbubble ultrasound derived blood flow parameters PI ratio and CE-HPI at 2 weeks after the beginning of the treatments to predict response correctly as indicated by RECIST at 3 months.
4.5.2 Cytotoxic chemotherapy

Thirty four patients were analysed and assessed for response in this study. Patients received chemotherapies: gemcitabine, cisplatin, 5-FU, oxaliplatin and irinotecan, in different combinations as a 1st line or 2nd line treatment (Appendix 5).

Figure 34. Patient recruitment summary. SD = stable disease, PD = progressive disease, PR = partial response. Patients with SD and PR were categorised as responders and PD as non-responders according to RECIST at 3 months. *Scan quality was audited by MA, who has 10 years’ experience of quantification of images.
Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with baseline 2 weeks scans, contrast scans and RECIST</td>
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</tr>
<tr>
<td>Mean Age</td>
<td>68</td>
</tr>
<tr>
<td>Age range</td>
<td>30-83</td>
</tr>
<tr>
<td>Patients with PS 2</td>
<td>10</td>
</tr>
<tr>
<td>Patients with PS 1</td>
<td>25</td>
</tr>
<tr>
<td>Males</td>
<td>23</td>
</tr>
<tr>
<td>Females</td>
<td>12</td>
</tr>
<tr>
<td>Patients with cholangiocarcinomas</td>
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</tr>
<tr>
<td>Patients with pancreatic cancer</td>
<td>6</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>16</td>
</tr>
<tr>
<td>Neuroendocrine tumours</td>
<td>5</td>
</tr>
<tr>
<td>Carcinoma of unknown primary</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 26. Summary of patient characteristics  
PS: Performance Status (Appendix 1)

Results

Peak intensity Ratio (PI HA/PIPV)

<table>
<thead>
<tr>
<th>PI ratio</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Responders (N=24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.74(4.7)</td>
</tr>
<tr>
<td>Non-Responders (N=11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.27 (3.3)</td>
</tr>
</tbody>
</table>

Table 27. Peak intensity ratio: Comparison between responders and non-responders. *statistically significant (Wilcoxon rank sum test p value < 0.05). The baseline comparison between two groups was statistically non-significant (Wilcoxon rank sum test p value > 0.05).

Decreased PI ratio in responders may indicate that vascular volume of the hepatic artery was reduced due to anti-vascular effects of cytotoxic chemotherapy. One plausible explanation for reduced PI ratio in responders could be reduced VEGF production by tumour cells as a result of cytotoxic effects of chemotherapy besides the direct killing of endothelial cells.
Figure 35. Peak intensity ratio: comparison between responders and non-responders. Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. The mean PI ratio was reduced (34%) significantly (p=0.01) in responders. Meanwhile, in non-responders the PI ratio had increased (i.e. increased vascular volume of hepatic artery as compared to the portal vein), which indicates increased arterial blood flow and progression in non-responders at 2 weeks.

### Contrast enhanced hepatic perfusion index (CE-HPI)

<table>
<thead>
<tr>
<th>CE-HPI</th>
<th>Baseline</th>
<th>2 weeks</th>
<th>Wilcoxon rank sum test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responders (N=24)</td>
<td>99.59(179.5)</td>
<td>64.3(187.3)</td>
<td>0.0049*</td>
</tr>
<tr>
<td>Non-Responders (N=11)</td>
<td>68.0(131.9)</td>
<td>366.8(544.1)</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

Table 28. CE-HPI: comparison between responders and non-responders  *statistically significant

(Wilcoxon rank sum test p value < 0.05). The baseline comparison between two groups was statistically non-significant (Wilcoxon rank sum test p value > 0.05).
Figure 36. CE-HPI: Comparison between responders and non-responders. Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. The mean CE-HPI was decreased (35%) in responders (p=0.0049) and increased in non-responders (p=0.02). The CE-HPI had decreased in responders, indicating a decrease in arterial recruitment and consequently volumetric blood flow in the hepatic artery as compared to the portal vein. Meanwhile, in non-responders CE-HPI had increased, indicating progressive arterialisation, leading to an increase in vascular volume of the hepatic artery.

Other blood flow parameters

PI of HA was reduced (p=0.11) while it had increased (p=0.019) in non-responders, which indicates increased arterial blood flow and progression in non-responders at 2 weeks. Other hepatic blood flow parameters such as RTHA, RTPV, and PIPV did not change significantly either in responders or non-responders. One possible reason could be the more obvious effect of antiangiogenic therapy on the arterial vascular volume, while there were subtle changes in the velocity of blood flow.
## Progression free survival and Overall Survival (PFS and OS)

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-Responders</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFS (days)</strong></td>
<td>241</td>
<td>108</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>OS (days)</strong></td>
<td>373</td>
<td>270</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 29: PFS and OS for responders and non-responders (according to CE-HPI ≥ 25% reduction) and non-responders (according to CE-HPI reduction <25 % or increase). Based on mean CE-HPI, PFS was 241 days in responders and 108 days in non-responders (hazard ratio 0.47 with 95 % confidence interval (0.23-0.97)). There was significance difference between responders and non-responders when log- rank test was applied (p=0.04). The OS was 373 days in responders and non-responders (270 days) and was statistically non-significant (p=0.06), when log- rank test applied (p=0.02) (hazard ratio 0.49 and 95 % confidence interval (0.22-1.1)). The possible reason may be that patients who progressed on previous treatments received a second line treatment and the effect was diluted.

### Discussion

Until now, no microbubble ultrasound study has conducted an investigation of global liver blood flow parameters to predict early response in patients with hepatobiliary and pancreatic cancer receiving cytotoxic chemotherapy, although many past studies have assessed the role of different biomarkers as potential predictive and prognostic tools in cancer management.

The role of VEGF levels as a biomarker has been widely investigated. Whilst high VEGF levels have been reported in different cancers (i.e. pancreas, CRC, cholangiocarcinomas) which were associated with poor prognosis, their role as a predictive biomarker remains unconfirmed[184].

Many studies have investigated Ca19-9 levels as a prognostic biomarker in pancreatic cancer patients with localised disease. Patients whose CA19-9 failed to return to the normal range...
after curative therapy had less than one year median survival as compared to median survival of more than one month for the group with normal range. Another study reported that patients who had > 75 % reduction in CA 19-9 levels after chemotherapy had median survival of 1 year in comparison to less than 3 months in non-responders to cytotoxic chemotherapy [185, 186].

KRAS (a type of protein involved in EGFR cellular signal transduction pathways) has been investigated as another potential biomarker for patients with CRC receiving anti-EGFR monoclonal antibody cetuximab. Patients with K-RAS mutant cancers had poor response to anti-epithelial growth factor receptor (EGFR) treatment. It was therefore considered as a useful prognostic biomarker for patients receiving anti-EGFR treatment [187].

Meanwhile, DCE-MRI has revealed significant reductions in vascular permeability and tumour perfusion (in terms of Ktrans and AUC60 ) in studies investigating antinagiogenic treatments (bevacizumab) with cytotoxic chemotherapies, as compared to taxanes alone. These results were significant given the fact that the two types of therapy have similar antivascular effects [188]. In rectal patients receiving chemoradiotherapy, tumours with relatively higher baseline blood flow were found to have poor response to treatment [189].

In studies investigating the role of FDG-PET as an imaging biomarker for antiangiogenic therapy, the standardized uptake values (SUVs) (ratio of tissue radioactivity concentration at the time of injection divided by body weight) have been shown to predict response in patients with lung cancer, thyroid cancer, lymphomas, prostate cancers and oesophageal carcinomas [190]. A review article by de Geus-Oei et al.(2007) revealed that SUVs in ^18^-F FDG PET were able more effectively to predict long term response in terms of PFS as compared to conventional anatomical criteria[191]. Although VEGF levels, KRAS, DCE-MRI and FDG-PET
have certain value as predictive biomarkers, these have not become popular, due to multiple issues of reproducibility, cost-effectiveness and validation[192].

The current study is the first to show possibility of reduced liver arterial blood flow happening as a result of cytotoxic chemotherapeutic agents in the gall bladder, pancreatic and colorectal cancers. One of the possible mechanisms could be reduced VEGF production as a result of cytotoxic effects on tumour cells, other than direct endothelial cell killing. It was also possible to predict responders against non-responders on the basis of microbubble ultrasound derived global liver blood flow parameters.

When the CE-HPI values were compared with conventional means of therapy assessment (RECIST based on gold standard CT scans), correlation was found between the two. Based on the interval change in CE-HPI and PI ratio at baseline and 2 weeks, an early response could be correctly predicted in 80 % of patients with liver tumours receiving cytotoxic chemotherapy and antiangiogenic drug combinations. The study shows that PI ratio and CE-HPI may be valuable tools for prognostic evaluation and treatment planning in cancer patients.

**Summary**

For the subgroup of patients who received cytotoxic chemotherapies, results showed the ability of microbubble ultrasound derived blood flow parameters PI ratio and CE-HPI at 2 weeks after the beginning of the treatments to predict response correctly as indicated by RECIST at 3 months. Correlation was also identified between CE-HPI and PFS and OS. This shows the potential of quantification analysis of global hepatic blood flow changes with
microbubble ultrasound derived blood flow parameters for early response assessment in patients with liver tumours receiving cytotoxic chemotherapy.
4.6 Title: Assessment of altered hepatic blood flow parameters in patients with liver tumours with selective interval radiotherapy treatment (SIRT)

**Background**

In the mid-1990s the use of microspheres containing yttrium$^{90}$ gamma particles was suggested for delivering high-dose internal radiotherapy to liver tumours [100]. Ionization radiation interacts at the cellular level with different cellular components, and causes DNA damage through formation of free radicals such as super oxide and hydrogen peroxide [102]. Besides inducing cell killing, this type of radiation therapy has been described to have a direct effect on tumour vasculature [103], through the radiation inhibiting ongoing angiogenesis, without affecting the established vasculature. This treatment, therefore, offers a dual benefit of micro-embolisation and high-dose radiation induced cell killing. SIR-spheres microspheres, when used in combination with chemotherapy or alone, provide an improvement in liver tumour control by shrinking the tumour and delaying progression in the liver[105].

Current response assessment methods have been limited in indicating the global liver blood flow changes in different phases and intervals; functional imaging such as microbubble ultrasound may offer an effective alternative for this purpose. The aim of this experiment was to quantify liver blood flow parameters in CRC patients with liver tumours being treated with SIRT, and to determine pre and post SIRT changes in arterialisation (of pre-angiography, pre-treatment, at 2 weeks and 6 weeks after SIRT) in terms of blood flow parameters.
Patient recruitment
This study was conducted from 1st March 2011 to June 2012; 31 patients were screened after obtaining their consent. Two patients did not receive SIRT because they did not satisfy the inclusion criteria (extra-hepatic shunting <20%) in angiography or other complications, so in total 29 patients were included.

Figure 37. Patient recruitment summary for SIRT (radio-embolisation). PD=progressive disease
SD=stable disease  PR=partial response. RECIST= response evaluation criteria in solid tumours
### Patient characteristics

| Number |
|------------------|-------------|
| Patients who completed scans, SIRT and RECIST | 25 |
| Males | 16 |
| Females | 9 |
| Mean Age (years) | 55 |
| Age range (years) | 30-75 |
| Patients with performance status (PS) 1 | 25 |

<table>
<thead>
<tr>
<th>Median PFS(days)</th>
<th>Median OS(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with PD</td>
<td>89</td>
</tr>
<tr>
<td>Patients with PR</td>
<td>259</td>
</tr>
<tr>
<td>Patients with SD</td>
<td>231</td>
</tr>
</tbody>
</table>

Table 30. Patients’ demographics at baseline.

PD=progressive disease, PR=partial response, SD=stable disease, PFS = progression free survival OS= Overall survival

### Methods

SonoVue preparation, method of injection, imaging technique, image transfer, quantification, image analysis and statistical analysis of blood flow parameters as described in methods chapter 3, pages 66-86.

### Protocol

The first step of procedure for SIRT is simulation, which includes performing a preliminary angiogram of the liver to determine the vascular anatomy. The femoral artery is then punctured through a French sheath catheter and French coaxial catheter, followed by
omnipaque-300 contrast injections into the coeliac axis, common hepatic, left and right hepatic and right gastric arteries. The gastroduodenal arteries (and optionally the right gastric artery/pancreatic duodenal branches) are embolised with multiple platinum coils to avoid deposition in the duodenum, stomach or pancreas. The macroagglutinated albumin (MAA) is delivered by separate injections through a coaxial catheter which is placed sequentially within the left and right hepatic arteries. MAA nuclear scanning is then performed to calculate the percentage dose of SIR-Spheres that will pass through the liver and lodge in the lungs or elsewhere due to arteriovenous shunting. The SIR-sphere dose is adjusted to limit radiation damage to the lungs. A dynamic contrast enhanced CT scan (DCE-CT) is performed to calculate the tumour percentage as a fraction of the whole liver, with the final microsphere dosage being decided on the basis of the percentage of shunting, the body surface area of the patient, and tumour percentage.

These patients were scanned prior to angiography, SIRT treatment, 2 weeks after SIRT treatment, and 6 weeks after SIRT treatment. Due to logistics and procedural reasons the following patients were not able to complete all scans (pre-angiography n=18, pre-sirtex n=23, 2 weeks after treatment n=18, and 6 weeks after treatment n=11).

Results

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>PA</td>
<td>pre-angiography</td>
</tr>
<tr>
<td>PS</td>
<td>pre SIRT</td>
</tr>
</tbody>
</table>

2weeks - 2 weeks after SIRT 6 weeks - 6 weeks after SIRT
Peak Intensity Ratio (PI HA / PI PV)

<table>
<thead>
<tr>
<th>PI ratio</th>
<th>Number of patients</th>
<th>Comparison with:</th>
<th>Mean (SD)</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-angiography</td>
<td>18</td>
<td>-</td>
<td>3.36 (4.43)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>Pre-SIRT</td>
<td>4.59 (5.3)</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2 weeks</td>
<td>5.86 (7.4)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6 weeks</td>
<td>2.6 (1.6)</td>
<td>0.38</td>
</tr>
<tr>
<td>Pre-SIRT</td>
<td>23</td>
<td>-</td>
<td>4.59 (5.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2 weeks</td>
<td>5.86 (7.4)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6 weeks</td>
<td>2.6 (1.6)</td>
<td>0.013*</td>
</tr>
</tbody>
</table>

Table 31. PI ratio for comparisons of effects of SIRT at different time intervals

*Statistically significant (Wilcoxon rank sum test p value < 0.05)

Figure 38. PI ratio for comparisons of effects of SIRT at different time intervals. Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. PI ratio is increased in post-angiography phase (albeit non-significantly), while reduced significantly at 6 weeks. The trend towards increase in the PI ratio at post angiography suggests the possibility of an increase in vascular volume and calibre of the hepatic artery as compared to the portal vein, based on liver haemodynamics haemostatsis principles.

One plausible explanation for the increased PI ratio at post angiography could be the excessive blood flow to the liver when the extra-hepatic branches (i.e. gastroduodenal arteries and optionally the right gastric artery/pancreatic duodenal branches) were blocked. However, at 6 weeks post treatment, the PI ratio was reduced significantly (p = 0.013),
indicating the decreased vascular volume of hepatic artery due to marked de-arterialisation and embolic effects in patients with liver tumours.

**Contrast Enhanced Hepatic Perfusion Index (CE-HPI)**

<table>
<thead>
<tr>
<th>CE-HPI</th>
<th>Number of patients</th>
<th>Comparison with:</th>
<th>Mean (SD)</th>
<th>Wilcoxon rank sum test (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-angiography</td>
<td>18</td>
<td>-</td>
<td>71.26 (200.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>Pre-SIRT</td>
<td>124.0 (333)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2 weeks</td>
<td>80.40 (156.1)</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6 weeks</td>
<td>18.35 (17.80)</td>
<td>0.54</td>
</tr>
<tr>
<td>Pre-SIRT</td>
<td>23</td>
<td>-</td>
<td>124.0 (333)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2 weeks</td>
<td>80.40 (156.1)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6 weeks</td>
<td>18.35 (17.80)</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

Table 32. **CE-HPI for comparisons of effects of SIRT at different time intervals** *statistically significant (Wilcoxon rank sum test p value < 0.05).*

**Figure 39. CE-HPI for comparisons of effects of SIRT at different time intervals.** Semi-logarithmic scale (log 10) is applied on y-axis to separate overlapped readings. Post angiography the CE-HPI (ratio of volumetric flow rate in HA to PV) shows an upward trend indicating possibly an increased blood volumetric flow in the hepatic artery as compared to the portal vein. This again might be consistent with the coil blocking of the extra-hepatic vessels. The CE-HPI is reduced significantly (0.02) at 6 weeks, which confirms the embolic effect of SIRT at this time interval, resulting in reduced volumetric flow in the hepatic artery as compared to the portal vein.
Other blood flow parameters

Rise time hepatic artery (RTHA) and RT PV which indicate the inverse of blood flow velocity did not change considerably at different time intervals, before and after angiography, SIRT, at 2 weeks and 6 weeks, indicating possibly the subtle changes in time based parameters. Peak Intensity HA (PIHA) which indicates vascular volume showed an upward trend from pre-angiography to pre-SIRT and then to 2 weeks; later it had reduced at the 6 weeks interval, approaching pre-SIRT level (albeit non-significant change). Pre-SIRT and post-angiography, the PIPV (vascular volume of portal vein) showed a downward trend but at 2 weeks it started rising, with a particularly marked increase at 6 weeks (p=0.004). This indicates that the portal flow increased significantly 6 weeks after treatment. As portal flow is compensatory, based on the haemodynamics of liver blood flow, it indirectly indicates a reduced volumetric arterial flow due to marked de-arterialisation at 6 weeks.

Discussion

The objective of this study was to assess haemodynamic changes in the liver due to SIRT intervention, as this treatment offers the dual benefits of micro-embolisation and high-dose interstitial radiotherapy.

Blocking extra hepatic vessels by platinum coiling before angiography leads to increased hepatic blood flow and this effect was also demonstrated in this study by the increase in the calibre of the hepatic artery and consequently the blood volumetric flow, reflected in the increased PI ratio and CE-HPI. At 2 weeks post-SIRT, evidence of increased arterial blood flow was found, reflected in a high PI ratio and CE-HPI, which could possibly have been due to inflammation or hyperaemia due to yttrium-90 particles. Blood inflammatory markers C-
reactive proteins may be helpful when tested before SIRT and at 2 weeks to confirm this finding. One possible implication of this finding would be that 2 weeks after SIRT would be the optimal time to add concurrent chemotherapy, as there would be more drug delivery to the tumour due to improved blood flow.

This is the first study to indicate that at 2 weeks there is more hyperaemic blood flow, possibly as a result of inflammation due to yttrium\textsuperscript{90} particles. Hence introduction of chemotherapy to the treatment, at this time point may help in improved drug delivery to the patient. The embolic effect of SIRT was found at 6 weeks, as indicated by decreased PIHA, PI ratio and CE-HPI, which is followed by a compensatory increase in portal flow (i.e. reduced portal hypertension). As a result, SIRT might be a better treatment in HCC patients with a background of cirrhosis and portal hypertension as such patients will tolerate SIRT better than other embolisation procedures, such as TACE [193, 194].

As TACE is contraindicated for treatment in primary liver cancer patients who have portal vein thrombosis, SIRT may be a better modality and more feasible for these patients, due to the continuous improvement in portal venous flow until 6 weeks after treatment, as demonstrated in this study [195].

Follow up of few patients participating in this study at 2 weeks and 6 weeks after SIRT has been limited due to logistical issues and the reduced number of patients at 2 and 6 weeks adversely affected the power of the study. In addition, the schedule of procedures for SIRT clashed with patients’ microbubble ultrasound appointments and often patients could not attend for that reason.
In this study population, most of the patients were having SIRT as a first line treatment, so their probability of being in the responders group was very high. Indeed, as was seen with the 3 month RECIST almost all of these patients were in the responders group. Hence, due to the absence of non-responders, comparison cannot be made between responders and non-responders to reflect any potential differences in terms of blood flow parameters.

**Summary**

With the help of microbubble ultrasound and global liver blood flow parameters, it was possible to determine the blood flow changes occurring in SIRT patients at different stages (i.e. before and after angiography, at 2 weeks and 6 weeks after SIRT). This is the first study to indicate that at 2 weeks there is a trend towards increased arterial blood flow, possibly as a result of inflammation due to yttrium90 particles. Increase in portal vein blood flow as a result of SIRT was also observed, which is perhaps why SIRT may be suitable for cirrhotic patients with HCC.
Chapter 5: Final Discussion and conclusion of thesis

Microbubble ultrasound has the advantage of microbubbles, as contrast agents, being purely intravascular without any extra vascular leakage, unlike contrast agents for CT scans and MRI [196]. Hence they can provide an accurate estimate of perfusion of blood vessels such as the hepatic artery and portal vein or individual hepatic tumours. With the development of hepatic tumours the normal liver haemodynamics- whereby the liver receives 30% of its blood supply through the hepatic artery and 70% through the portal vein- is altered, as liver tumours derive the majority of their blood supply (about 90%) from the hepatic artery, resulting in hyper-arterialisation. This hyperarterialisation can be assessed through microbubble ultrasound derived global liver blood flow parameters derived from the hepatic artery and portal vein.

The working hypothesis of this thesis was that assessment of global liver blood flow by a microbubble ultrasound derived hepatic index known as CE-HPI can predict an early response to cancer treatment.

We proposed that in patients who do not respond (on 3 months RECIST), this index may increase from baseline or remain static as the liver tumours continue to arterialise or do not de-arterialise at all; whereas in patients who are responders at 3 months, the index may decrease at 2 weeks compared to baseline, as the liver tumours may de-arterialise at 2 weeks. Based on this assumption, the index may potentially predict response to treatment earlier (at 2 weeks) as compared to conventional imaging methods based on RECIST.

To test this hypothesis, this thesis addressed the following questions:-

1. Does the index CE-HPI derived from liver blood flow parameters depict liver parameters such as volumetric flow rate and velocity in vitro (phantom)? As there is a lack of experimental data to establish the relationship between microbubble ultrasound
derived blood flow parameters and physiological characteristics of liver blood flow such as volumetric flow rate and velocity, in the first experiment, we investigated the relationship between the CE-HPI and physiological characteristics, such as volumetric flow rate, in a phantom. In this experiment CE-HPI values were determined for different volumetric flow rates. It was found that when the volumetric flow rate was increased, the CE-HPI values increased and vice versa.

2. Is CE-HPI different in normal (healthy) persons as compared to patients with liver tumours, and what is the range? To answer this question, contrast agents were injected in healthy volunteers and patients and CE-HPI values were quantified and compared in both sets of patients. Increased CE-HPI values in patients indicate increased arterialisation, as compared to healthy individuals. As a secondary finding, peak intensity ratios (PI ratios) were also found to be significantly higher in patients with liver tumours at baseline without any treatment, as compared to healthy individuals.

3. In order to compare meaningful changes in patients receiving treatments, are these CE-HPI values reproducible for inter-operator, inter-scan, fasting vs. non-fasting and variability on Q-lab quantification software, and what is the COV? In the 3rd experiment, inter-operator, inter-scan, fasting vs. non-fasting variability and quantification variability were studied for CE-HPI, and the COV for CE-HPI was found to be around 40% (and 20% for PI ratio), which was comparable to other imaging techniques such as DCE-MRI [165].

4. Can CE-HPI predict response to treatment earlier (at 2 weeks) than standard imaging techniques (at 3 months) in patients with liver tumours receiving either cytotoxic chemotherapy alone or in combination with biological agents? In the next experiment, for response assessment, one group was treated with a combination of systemic
cytotoxic chemotherapy and anti-angiogenic therapy and the second group with chemotherapy alone. The main objective of this experiment was to determine whether the index CE-HPI derived from global liver blood flow parameters for the hepatic artery and portal vein could help with prediction of response in patients with liver metastases and primary liver cancer, receiving either chemotherapeutic agents alone or in combination with antiangiogenic biological agents. Patients in the non-responder group (response assessed on 3 months RECIST) either had progressive arterialisation despite treatment or no significant change in arterialisation as depicted in volumetric arterial blood flow indicated by increased CE-HPI (p=0.04) and PI ratio (p=0.03)(table 19 and 20). The PFS and OS (table 21) were significantly different between the two groups, with hazard ratios of 0.25 to 0.45. Comparison of Kaplan-Meier survival curves between CE-HPI responders and CE-HPI non-responders demonstrated statistical significance for PFS, whilst Kaplan-Meier survival curves for the subgroups of cytotoxic chemotherapy and antiangiogenic treatments did not demonstrate significance.

One plausible explanation could be that these patients had received further second line treatments following progression. This is the first study indicating de-arterialisation due to cytotoxic chemotherapy as well as biological agents in patients with primary and secondary liver cancers, with the potential to predict responders’ against non-responders’ status on the basis of global liver blood flow parameters and global index CE-HPI.
Table 33. Summary of the thesis findings: Percentage change in hepatic blood flow parameters at 2 weeks. Patients were grouped into responders (decrease in CE-HPI of > 25%) and non-responders (percentage reduction in CE-HPI of < 25% or increase). *Statistically significant at p < 0.05 Wilcoxon rank sum test † Change at 6 weeks.

These significant findings in the study may indicate that CE-HPI could be a good biomarker of response regardless of different tumour groups receiving different treatments (table 34).

Table 34. Summary of the study findings: Progression free survival and overall survival

PFS: progression free survival OS: overall survival * statistically significant Kaplan-Meier survival curve p value <0.05

5. And finally, can CE-HPI depict changes in liver blood flow at different time intervals (before angiography, at SIRT treatment, 2 weeks, and 6 weeks after treatment) in
patients receiving selective internal radiation therapy (SIRT)? In the last experiment the global index CE-HPI was found to have increased before the SIRT procedure, which possibly indicated increased hepatic arterial volumetric flow due to blockage of shunts, whereas at two weeks after the SIRT procedure, a further increase in CE-HPI probably indicated hyperaemia due to yttrium gamma radiation therapy. Finally, at 6 weeks, there was a decrease in CE-HPI values indicating decreased arterialisation and arterial shutdown due to embolic and cytotoxic effects of gamma radiation.

The final conclusion was that the global index CE-HPI not only has the potential to predict response to treatment at 2 weeks, but also as an index it has been shown to be effective regardless of therapy and with different primary cancers in patients with liver tumours, with reproducibility comparable to other techniques.

It has the further advantage of being easier to set up in outpatient departments, with mobile ultrasound scanners being operated by trained operators, after a learning curve of 3-6 months. If the findings on baseline analysis can be confirmed in other centres, and this index is adopted, patients could avoid costly and toxic antiangiogenic drug combinations and could be given alternative treatments, including ablation techniques.

As microbubble ultrasound could potentially predict an early response in patients with primary and secondary liver cancer, it can act as a surrogate imaging biomarker for early response assessment in tumour groups receiving chemotherapy, as mentioned above.
Comparison with past studies

This study is unique from the previously mentioned studies in certain aspects, with various pros and cons. First, most of the previous studies were based on tumour perfusion, whereby time intensity curves were derived from the tumour, after injection of a contrast agent. The previous research groups (Lassau et al., Williams et al., and Averkiou et al.) selected the index lesion(s) (one or two) in the liver for measuring tumour perfusion before and after treatment, at different time intervals [14, 136, 138]. They then compared the response of these index lesions with the RECIST, progression free survival (PFS), and overall survival (OS). A potential problem with this technique is that individual lesions within the liver can exhibit different biological behaviour and it is unknown at presentation which index lesion, over time, is going to be representative of response. Also the liver micrometastases are not identified by contrast scans; hence, as vascularity of these micrometastases inside the liver is very difficult to assess, it may not be realistic to measure tumour perfusion for individual metastases. In this thesis, blood flow changes were examined throughout the liver, and global blood flow parameters were derived from the TICs extracted from the hepatic artery and portal vein. Microbubble ultrasound derived blood flow parameters can help identify subtle changes in the perfusion of micrometastases and be helpful for follow ups after the assessment of response.

As mentioned earlier in relation to the tumour perfusion studies, the researchers Lassau et al. (2011) and Williams et al. (2011) investigated multiple parameters, such as rise time (RT), time to peak intensity (TPI), wash in slope (WIS), peak intensity (PI), area under the curve (AUC), area under wash in (AUWI), area under wash out (AUWO), wash out slope (WOS) and mean transit time (MTT), for early response assessment. Each of these parameters represents one aspect of tumour perfusion rather than the whole tumour perfusion process.
or volumetric flow. In contrast, the current study focused on the volumetric blood flow index (CE-HPI), which is more representative of blood flow changes happening inside the liver than are the individual perfusion parameters of an index lesion. Therefore, the CE-HPI may be a better predictor than the 7 separate parameters used to derive tumour perfusion, which are at higher risk of statistical errors.

Another issue not addressed in the previous tumour perfusion studies was the many uncertainties associated with bolus injections, especially in relation to the input function in the region of interest (tumour or vessel) and the variations induced by different volumes and rates of contrast injection by different operators. To overcome these uncertainties, normalisation was used by obtaining the ratio of blood flow factor derived from the hepatic artery to those in the portal vein. Normalisation enabled more consistent and reliable results to be achieved through removal of the uncertainties induced by multiple injections and the undefined input function during bolus injection. In general, tumour perfusion studies have not used the above process of normalisation, except for the work of Averkiou et al. (2010 )[14]. Another implication of this study could be that patients with hypovascular tumours and low CE-HPI values at baseline may be selectively treated with ablation techniques rather than being exposed to toxic chemotherapeutic and antiangiogenic drug combinations.

The CE-HPI as a global index could play a potential role in the non-invasive assessment of tumour angiogenesis. Microvessel density (MVD) is a measure of angiogenesis, and a reference standard in the quantification of blood vessels [197, 198]. Normally it can only be assessed in surgical biopsy and histopathology specimens, which as invasive procedures are associated with complications [199]. Several microbubble ultrasound studies have already
been conducted into non-invasive assessment of tumour angiogenesis. Wang et al. (2007) performed a microbubble ultrasound tumour perfusion study of 50 patients with HCC to correlate the tumour perfusion parameter PI (which indicates vascular volume) with the MVD of the tumour [200]. After determining the tumour perfusion parameter PI, they performed immunohistochemistry to analyse the MVD in the corresponding tumour specimens. They not only found values of PI and MVD in HCC to be higher than in the surrounding non-cancerous parenchyma, but also found a significant correlation between PI and MVD in HCC tissues [200]. A further microbubble ultrasound study of tumour perfusion by Zhuang et al. (2012), this time in patients with CRC, studied correlation between time intensity curve parameters and expression of VEGF or MVD [201]. In this study, the researchers found that both AUC and MVD were significantly higher in colorectal adenocarcinomas as compared to adenomas (all P < 0.05). A positive linear correlation was also found between AUC and MVD in colorectal tumours (r = 0.686, P = 0.0019), whilst no correlation was found between VEGF and any other tumour perfusion parameter. Correlation of MVD and VEGF levels with microbubble ultrasound derived blood flow parameters would be the next logical step for future research in microbubble ultrasound [201].

**Challenges, limitations and potential solutions**

Due to author of this thesis being a non-radiologist, 40% of the scans initially were discarded by the independent reviewer (MA) due to inadequate quality of the scans. Auditing of images was performed independently by MA, who has 10 years’ experience of quantification analysis of images.
Three operators, AM (author of this thesis, experience of 100 scans at the start of the project), XF (10 years’ contrast scan experience) and YZ (7 years’ contrast scan experience), performed the scans for the patients in this study. Individual inter-operator variability among these three might be a concern, although it was found that inter-operator variability for the PI ratio and other individual parameters was 20 %, which was comparable to other reproducibility studies[14, 27, 174].

In obtaining the sample for this study, non-probability sampling technique was used for patient recruitment. As there was a heterogeneous mix of tumour types and stages, with heterogeneous chemotherapeutic regimes, this adversely affected the power of the study. Phase III studies, with randomization, need to be conducted at different centres and other tumour groups need to be analysed with microbubble ultrasound.

The limited number of patients involved in the reproducibility study, due to logistical problems, could be a potential problem. Another major factor has been the loss of patients due to treatment complications; primary liver cancer patients, in particular, faced many complications during the first few weeks, which made it difficult to scan them at an early point. The patients with HCC (n=6) were lost due to comorbidities and treatment complications, making the final sample size quite small, which again affected the power of the study. One of the other most common reasons for exclusion of patients from this study project was obesity, and patients with obese build also contributed to poor quality images which were rejected on independent review by MA.

Another problem encountered with obese patients is that a low mechanical index (MI) gives poor depth penetration and visualization of vessels where the portal veins are deep. On the other hand, a high mechanical index of >0.06, which disrupts microbubbles, can allow the
deep portal veins to be visualized better but this results in deviation from the standard protocol.

In this thesis, loops for the time intensity curves (TIC) were captured for only 60-70 seconds, with emphasis on wash in parameters only, such as wash in time / rise time, peak intensity and wash in slope. The exclusion of wash out parameters (MTT and AUC) might be a limitation of the study. With long loops (>1 minutes), the patient’s breathing becomes irregular, which affects the quality of imaging. The patients were not given any instruction about breath holding but instead were allowed to breathe freely and later respiratory gating technique was utilized to remove out of plane images [14]. Lassau et al. (2010) also obtained the TIC without breath holding, but did not use any respiratory gating or breathing compensation techniques [136]. However, in the current study without respiratory gating technique, loops had too much noise for analysis. A mechanical arm which could hold and fix the probe to the patient has also been suggested as a means to reduce hand motion.

A related challenge is that recirculation of the contrast agent occurs at around 40-45 seconds, which dilutes the wash out parameters, including the area under the curve. Also it is quite difficult to maintain the same imaging plane for more than 60 seconds. Lassau et al. (2010) maintained the TIC for a period of 3 minutes, and did not describe any method to counter recirculation [136]. One major reason for excluding wash out parameters in this study has been the re-circulation of the contrast agent, coming in around 40 seconds after the start of image loop[202]. Quantification analysis of the recirculation component has been extremely tricky and no reasonable model has so far been suggested [14].

Meanwhile, motion compensation (in one plane) on image analysis was one of the limitations of our analysis. As the scans are 2-dimensional, image plane compensation is also
in 2 planes. However, 2-dimensional planes may not always be representative of the whole liver tumour, and they are difficult to maintain for follow up scans and serial monitoring of treatment. With 3-dimensional probes tumour microcirculation can be imaged and analysed in multiple planes, therefore issues of motion compensation in all 3 image planes might be resolved.

Another challenge has been maintaining the same image scan plane for the hepatic artery and portal vein at different time intervals, when scanning the patients. Even a slight change in the cross sectional imaging plane can affect intensity values on microbubble ultrasound derived TIC. Watching the previous image loops of the particular patients was helpful in maintaining the same plane before the next scan.

As microbubbles do not extravasate, microbubble US cannot alone provide information about the vascular permeability of tumours which could in turn give us important information about treatment resistance due to poor delivery of oxygen and cytotoxic chemotherapeutic drugs. DCE-CT and DCE-MRI are better modalities for gaining such vascular permeability information.

Although different models have been suggested on the basis of indicator dilution theory, Averkiou et al. (2010) propose the local density random walk model (LDRW) and lognormal wash in wash out model (LNWIWO) for quantification of liver blood flow parameters, because their physiological and physical basis takes into consideration the architecture of microvasculature[14, 61].

Another concern in relation to this study was the quality of the contrast agent (SonoVue) solution after preparation. According to manufacturer instructions, SonoVue must be used...
within 6 hours of constitution, when used for diagnostic purposes. Soon after solution preparation, natural deterioration (unlike ultrasound induced) of the microbubbles occurs which may affect the quality of the experiment and quantification analysis. To counter this, image loops were acquired within 10 minutes of solution preparation.

Variation in the rate of contrast agent administration is another limiting factor. Patients in the oncology clinic on 2nd line and 3rd line treatments have poor venous access and there is difficulty in 20 Gauge cannulation, so in consequence microbubbles injected through small bore cannulas might get destroyed. Hence, these patients were also excluded from the final assessment as a quality assurance measure.

It is also possible for contrast injections to have a confounding effect on subsequent measurements. This may be due to the effect of contrast take up in the vascular system and endothelium vessel wall and recirculation of residual contrast[134]. In the current thesis, a break of at least 10 minutes was allowed between different injections, to avoid this unwanted eventuality.

A further concern in this study was the dose of the contrast agent. Initially 2 mls of contrast agent was used, but later this was replaced with a reduced 1.2 ml dose (for experiment 4.6), which was found to be sufficient for contrast enhancement of hepatic artery and portal vein. In contrast, studies (tumour perfusion) by other groups have used high doses of up to 4.8 mls [136, 138].

Bolus vs. disruption replenishment technique is another major issue in terms of microbubble administration (described in chapter 2.1.6 page 38). Most of the tumour perfusion studies have used bolus technique for contrast injection, except for Williams et al., who
simultaneously performed the study with infusion of the contrast injection by using the disruption replenishment technique[138]. This technique has certain advantages, such as many planes can be scanned, and also uncertainty of the input function of the contrast at ROI is reduced. But at the same time the disruption replenishment technique requires expensive specialised equipment, which is usually not available at the bedside. Also the quantification techniques for infusion and disruption replenishment methods are more complicated and still in the early stages of development.

There are also challenges associated with the implementation of microbubble ultrasound at institution level, which limit the widespread use of quantification techniques in routine clinical practice. PET-CT, DCE-MRI and DCE-CT are the most commonly used investigation modalities in cancer centres. Most oncologists are unaware of the value of microbubble ultrasound and consequently the use of microbubble ultrasound for real time functional assessment of response to antiangiogenic therapy is still in the early stages in most clinics [203]. Awareness has to be generated regarding the qualitative and quantitative potential of microbubble ultrasound through international forums. Education and training of personnel in microbubble ultrasound and publication of consensus guidelines is the need of the hour. Meanwhile, the financial implications of microbubble ultrasound are another huge concern for the majority of institutions worldwide [150].

**Practical applications of this work**

Microbubble ultrasound analysis of global liver blood flow parameters may be an alternative for response assessment in patients with liver metastases and primary liver cancers. By identification of resistant patients at an early time point, inappropriate treatments can be
stopped and patients can switch to more effective therapy. This can help in saving costs as well as minimizing toxicities in cancer patients.

Microbubble ultrasound is quantitative in nature and thus more objective. Before and after treatment, analysis with microbubble US technique also allows evaluation with reference to baseline state, thus decreasing intra-individual variability.

Quantification protocols and imaging techniques used in this thesis may be easily implemented in routine clinical practice, especially in outpatient departments. Plotting of the time intensity curve (TIC) and calculation of blood flow parameters can be performed automatically by software packages available on some US systems. This can help to achieve bedside portability of contrast scans in oncology departments, where patients at their follow up could undergo contrast ultrasound and the physician could see early changes in liver haemodynamics due to response or non-response.

Microbubble ultrasound also has the advantage of being non-ionizing and not exposing patients to the potentially harmful contrast agents used in other imaging modalities.

The hepatic blood flow parameters, especially the PI ratio and CE-HPI, can be used for staging work up of patients with early stage tumours to find out if any adjuvant treatment is indicated, which can improve the PFS and OS. These blood flow parameters can also be helpful in long term follow up of patients. At the same time, early diagnosis of patients can help improve the survival outcome by giving patients the best possible cytotoxic chemotherapy and biological agents, alone or in combinations.

Microbubble ultrasound can, in addition, be repeated multiple times without any side effects. And tumour perfusion studies conducted in the past have shown their potential as
an imaging biomarker for predicting early response. These studies indicate that microbubble ultrasound could be used for investigating pharmacodynamics and pharmacokinetic properties of new antiangiogenic biological agents.

Microbubbles are pure blood pool contrast agents without any extravasations, whereas contrast agents for DCE-MRI and DCE-CT do extravasate and give information about vascular and tumour permeability, and may thus be used to complement information from microbubble ultrasound. Further studies need to be conducted in which several such imaging modalities are combined to compare the reproducibility of derived quantitative blood flow parameters and also to predict the efficacy of multiple imaging biomarkers[203].

In clinical research, treatment end points such as PFS and OS require quite lengthy follow-ups, which can prove too costly and have associated logistic problems. However, microbubble ultrasound as an early response imaging biomarker has the potential to overcome these challenges [203].

The ultimate aim of this thesis work was to identify a method for early assessment of tumour response to chemotherapy and biological treatments and also to determine whether these results are reliable. The CE-HPI and PI ratio derived from microbubble ultrasound helped in correct prediction of early response in 80 % of patients in this study population. Hence, these liver blood flow parameters have the potential to act as an early response biomarker.
Summary and conclusion

As a non-invasive imaging biomarker, microbubble ultrasound can be used to assess global liver blood flow in patients with liver tumours through microbubble ultrasound derived blood flow parameters, especially the PI ratio and CE-HPI. Also this method is reproducible, so the predictive value of microbubble ultrasound derived blood flow parameters, particularly CE-HPI, for treatment response could act a potential biomarker of treatment outcome.

Future Work

This study has provided a non-invasive assessment of tumour vascularity in terms of global liver blood flow parameters. The accuracy of this study should be confirmed by other minimally invasive and invasive biomarkers such as mean vascular density, VEGF derived from histopathology and VEGF serum levels. In a future study, the correlation of microbubble ultrasound derived global liver blood flow parameters with mean vascular density, VEGF derived from histopathology and VEGF serum levels, should be determined.
References


80. Hudson, J.M., Quantification of blood flow using ultrasound contrast agents, in Department of Medical Biophysics 2011, University of Toronto: Toronto. p. 178.


171
early evaluation of antiangiogenic treatment. Lassau, N., et al.,
evaluation.
Ramnarine, K.V., et al.,
1,081 transplanted livers.
Lopez colorectal metastases.
Roumen, R.M., et al.,
Glover, C., et al.,
Results of a survey of members of the American Society of Colon and Rectal Surgeons.
Vernava, A.M., 3rd, et al.,


Appendix 1: Performance Status (PS)

0 – Fully active and more or less as same as before illness

1 – Cannot carry out heavy physical work, but can do anything else

2 – Up and about > 50 % of the day and can look after himself/herself, but not well enough to work

3 – In bed or sitting in a chair for > 50 % day and needs some help in looking after himself/herself

4 – In bed or a chair all the time and need a lot of care
Appendix 2: Important functions on QLAB Software

Uncompressed linear data are used for the analysis, as log compression results in loss of useful data and is not fit for quantification purposes. Regions of interest (ROI) of equivalent sizes are drawn over the HA and PV lumens. Following are the few steps and important functions on QLAB software.

Figure 1. On desktop, bring cursor to QLAB and double click on it.

Figure 2. QLAB software opens and first interface appears.
Figure 3. Find the folder with saved loops on left side.

As this quantification software is only for experimental use, it clearly says work in progress and not for standard diagnostic use.

Figure 4. Bringing the cursor onto folder sign: says open a file
Figure 5. Shows different hard drives go to the hard drive with the saved data.

Figure 6. Shows anonymised list of patients on left side. Click on the concerned patient data file.

It opens like this. A shows hepatic artery and portal vein in B-mode, shows Doppler scans on B, C, and D.
Figure 7. Screen shot after clicking the desired loop.

Showing colour / contrast mode on left side and brightness mode on right side

---

Figure 8. By clicking on the concerned loop, screen appears with the functions on the left hand side, and also on the bottom of the two images.
Figure 9. Important functions on QLAB
Figure 10. First and last frames.

Select the first and last frames by clicking on the tabs as indicated above and then play the loop. It will show the contrast mode on the right side and brightness mode on the left side. The diaphragm as bright reflector will show up in brightness mode, while in contrast mode, hepatic artery and portal vein can be seen being filled with contrast microbubbles at about 15 sec and 35-40 seconds respectively.
Appendix 3: QLAB Protocol [204]

“Transferring data from IU-22 to Hard Drive

(1) Select Review from Control Panel

(2) Select the exams you want to transfer

(3) Select Extract data

Transferring data from Hard Drive to Computer

Note: Every time data is transferred to the hard drive it is transferred in a folder named DICOM so make sure that you date the folder with the date of transfer (ex. 2014xxxx DICOM) and move the folder to IU-22 Backup before transferring any more data from the IU -22 machine otherwise you will overwrite the previous data.

(1) Create a folder with the patients Name and Treatment

(2) Create subfolders with the data of the scans and add the required DICOM files

(3) Create a word document for each folder to keep a record of the analysis and print screen the results example:

Date of scan and type of treatment

Hepatic artery (HA)

Portal vein (PV)

Tumour

Liver

(a) QLAB Analysis

(1) Open QLAB

(2) Select the folder you want to work on from the selection list on the left of the screen
(3) For Doppler perfusion index (DPI) measurements:

- Calculate the average of the Velocity and Area for the HA and PV

(4) For Time Intensity Curves (TIC) measurements of the HA, PV, Tumour and Normal Liver Parenchyma

- Select the loop you want to analyze
- Before starting the analysis scroll though the loop in order to identify the basic structures and anatomical landmarks (diaphragm or other bright landmarks)

**For HA and PV analyses**

(a) Select a frame that indicates the HA and PV clearly on the **contrast image** preferable during the arterial phase and make a note of the **reference frame number**

(b) From the **tool box** on the left of the screen select the **ROI tool → Freeform Spline** and draw a region of interest focusing on the HA lumen in order to generate the curve. **Zoom in** to make the visualization of the structures more clear

(c) Scroll through the loop to monitor the pattern of the curve and to make sure that your ROI is around the HA during most of the frames. **Zoom out.**

(d) Return to your **reference frame number** and repeat steps b – c for the PV

(e) From the **tool box** select the LDRWWIWO curve option to normalize the data

***Note: To remove the LDRWWIWO curve from the tool box select clear***
(f) Return to your reference frame number and draw an ROI around the diaphragm or other bright anatomical landmark on the B-mode image.

(g) After the curve is generated for the diaphragm/ bright anatomical landmark return to the reference frame and select the Filter option from the tool box in order to perform respiratory gating.

***Note: Respiratory gating is performed based on the ROI of the selected structure. ALWAYS perform respiratory gating with the ROI selected for the generated by the diaphragm/ bright anatomical landmark.

(h) In the filter window that comes up on the screen type 0.3 (in some cases you made need to choose 0.2 or 0.5 depending on the quality of the images and the pattern of the curve generated by the diaphragm/ bright anatomical landmark ) and select ok.

***Note: Even if you select cancel option respiratory gating will still be applied. *** Note: After respiratory gating is performed if you are not happy with the outcome of the curves select the icon to bring back all the frames *** QLAB has no UNDO selection so if not happy with your analysis you need to delete you ROIs and start from the beginning.

(i) After respiratory gating is performed scroll through the entire loop to make sure that your ROI’s on the HA and PV are on the specific structure at all times (in most cases the ROI is not in place at all times due to the patients respiratory motion). You will then need to select your reference frame or other frame that indicates your ROI completely around your structure that you need to focus on (HA or PV) and select from the tool box the Active motion compensation option. 

Active Motion
**Compensation** is used when necessary if respiratory motion entails further correction

*** Note: Active motion compensation is not performed on all structures at once so you need to do it independently for each structure when needed

(j) If a number of frames is still out of plane you will need to manually adjust them by using **Create new frame** from the **tool box**

(k) When finished with the analysis select the **Save** option from the **tool box** and save your analysis

*** Note: It is a good idea to start saving your analysis even during earlier steps

(l) Perform the above steps for the tumour and normal liver parenchyma

*** Note: The reference frame for the tumour does not have to be in the arterial phase. Select according to the discretion of the investigator

*** Note: Try to draw the liver parenchyma ROI on the same level as the tumour (medial or lateral) if possible” [204-206]
### Appendix 4: Comparison of Imaging Techniques for Quantification of Blood Flow

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Microbubble ultrasound</th>
<th>DCE-CT</th>
<th>DCE-MRI</th>
<th>0-15 PET</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signal in relation to enhancement of tumour</strong></td>
<td>Linear</td>
<td>Linear</td>
<td>Complex</td>
<td>Linear</td>
</tr>
<tr>
<td><strong>Temporal resolution of less than 1 sec</strong></td>
<td>Possible</td>
<td>Possible</td>
<td>Not possible</td>
<td>Possible</td>
</tr>
<tr>
<td><strong>Area covered</strong></td>
<td>1 slice</td>
<td>Up to 4 cm</td>
<td>Large area</td>
<td>Large area</td>
</tr>
<tr>
<td><strong>Type of study</strong></td>
<td>First pass</td>
<td>First pass and permeability</td>
<td>First pass and permeability</td>
<td>First pass</td>
</tr>
<tr>
<td><strong>Respiratory misregistration</strong></td>
<td>Affects study</td>
<td>Affects study</td>
<td>Affects study</td>
<td>Affects study</td>
</tr>
<tr>
<td><strong>Repeatability</strong> (more number of studies within 24 h)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Spatial Resolution</strong></td>
<td>300-500 μm</td>
<td>0.5 mm</td>
<td>1 mm</td>
<td>1-2 mm</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Real time</td>
<td>Simple quantification</td>
<td>Non irradiant Soft tissue contrast</td>
<td>Excellent sensitivity</td>
</tr>
<tr>
<td></td>
<td>Non irradiant</td>
<td>Diffusible contrast agent</td>
<td>Diffusible contrast agent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intravascular contrast agent</td>
<td>Linear contrast agent</td>
<td>Published best practice guidelines</td>
<td></td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Limited penetration</td>
<td>Radiation exposure</td>
<td>Low frame rate</td>
<td>Irradiant Short tracer half life</td>
</tr>
<tr>
<td></td>
<td>operator dependent</td>
<td>Contrast agent toxicity</td>
<td>Long procedure time</td>
<td>Costly Limited availability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffusible contrast agent</td>
<td>Costly</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Commercially available</td>
<td>Motion artifact</td>
<td>Lack of anatomical information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>analysis software</td>
<td>Lack of uniform software</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/ analysis algorithms</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical validation</strong></td>
<td>-</td>
<td>Correlation with MVD and PET</td>
<td>Correlation with MVD, tumour hypoxia, and VEGF expression</td>
<td></td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td>-</td>
<td>Reproducible</td>
<td>Difficult to correlate between centres</td>
<td>Most reproducible technique</td>
</tr>
</tbody>
</table>

Table: Comparison of imaging techniques for quantification of blood flow. Adapted from [25-28]
Appendix 5: Cytotoxic chemotherapeutic regimens

1. **DeGramont – Modified**
   - Folinic Acid 350 mg IV over 2 hours Day 1
   - 5 Fluorouracil 400 mg/m² IV bolus Day 1
   - 5 Fluorouracil 2800 mg/m² IV over 46 hours Days 1 to 2
   - Repeated every 14 days, 6-12 cycles in 3-6 months for hepatobiliary cancers

2. **Gemcitabine-Cisplatin**
   - Pre-hydration Day 1
   - Cisplatin 60mg/m² IV over 2hrs Day 1
   - Gemcitabine 1000mg/m² IV over 30mins Days 1 and 8
   - Post-hydration Day 1, repeated every 21 days, 4-8 cycles for cholangiocarcinoma or pancreatic cancer in 1st line.

3. **Gemcitabine 1+8+15**
   - Gemcitabine 1000mg/m² IVI over 30mins Days 1,8,15, repeated every 28 days
   - Treatment may start with weekly chemotherapy for 7 weeks i.e. days 1,8,15,22,29,36, 43, then one week off, then treatment follows day 1,8,15 repeated days 28, 6 cycles for adjuvant or palliative pancreatic cancer:

4. **Capecitabine 2500 (Degramont substitute)**
   - Capecitabine 1250mg/m² BD Oral Days 1 to 14 after food with water
   - If CrCl >50mls/min standard dose, starting dose reduced by 25% for age>70 years
   - Repeated every 21 days, -8 cycles/3-6 months for 1st line metastatic colorectal cancer

5. **Tegafur with Uracil (Uftoral)**
   - Uft oral 324mg/m² TDS Days 1 to 28
   - Calcium folinate 30mg TDS Days 1 to 28 (Folinic acid)
   - Uft oral 972mg/m²/day = tegafur 300mg/m²/day and uracil 672mg/m²/day.

Each Uftoral capsule 324mg contains Tegafur 100mg and Uracil 224mg Repeated every 35 days, for metastatic colorectal cancer 1st line only 2-5 cycles/3-6 months depending on response (tumour markers and CT scans).
<table>
<thead>
<tr>
<th>Surface area $m^2$</th>
<th>Uft oral capsules/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1.17</td>
<td>3 capsules/day</td>
</tr>
<tr>
<td>1.17-1.49</td>
<td>4 capsules/day</td>
</tr>
<tr>
<td>1.50-1.83</td>
<td>5 capsules/day</td>
</tr>
<tr>
<td>&gt; 1.83</td>
<td>6 capsules/day</td>
</tr>
</tbody>
</table>

Table. UFT oral capsules doses. Adapted from [207]

6. **Mdg plus Irinotecan**
   Atropine 250mcg SC Day 1
   Irinotecan 180mg/m2 IV over 30-90mins Day 1
   Folinic Acid 350mg IV over 2 hours Day 1
   5-Fluorouracil 400mg/m2 IV bolus Day 1
   5-Fluorouracil 2400mg/m2 IV over 46 hours Day 1 to 2
   Repeated every 14 days, and for colorectal cancer metastatic: 6-12 cycles/3-6 months

7. **Irinotecan-Capecitabine (Capiri)**
   Atropine 250mcg SC Day 1
   Irinotecan 200mg/m2 IV over 30-90mins Day 1
   Capecitabine 800mg/m2 Oral twice a day 1 to 14 with water after food
   Repeated every 21 days, and for colorectal cancer 1st line metastatic 4-8 cycles/3-6 months

8. **OxMdG DeGramont – Modified plus Oxaliplatin**
   Folinic Acid 350mg IV over 2 hours Day 1
   Oxaliplatin 85mg/m2 IV over 2 hours Day 1
   5-Fluorouracil 400mg/m2 IV bolus Day 1
   5-Fluorouracil 2400mg/m2 IV over 46 hours Day 1 to 2
   Repeat every 14 days, 6-12 cycles/3-6 months for 1st or 2nd line metastatic colorectal cancer.

9. **Oxaliplatin-Capecitabine**
   Oxaliplatin 130mg/m2 IV over 2 hours Day 1
   Capecitabine 1000mg/m2 orally twice a day, Day 1 to 14 after a meal with water
   Repeated every 21 days
Biological Agents alone and in combinations

1. Cetuximab Monotherapy/Single Agent weekly
   Cycle 1, loading dose Cetuximab
   Chlorphenamine 10mg IV bolus dose Day 1
   Dexamethasone 8mg IV bolus dose Day 1
   Cetuximab 400mg/m² IV over 2 hours once only day 1
   Cycle 2 onwards
   Maintenance dose Cetuximab
   Chlorphenamine 10mg IV bolus dose Day 1
   Dexamethasone 8mg IV bolus dose Day 1
   Cetuximab 250mg/m² IV over 1 hour* Day 1
   Repeated every 7 days, loading dose cetuximab 400mg/m² once only week one
   followed 7 days later by cetuximab 250mg/m² repeated every 7 Days thereafter,
   for EGFR expressing KRAS wild type metastatic colorectal cancer

2. Bevacizumab-Oxaliplatin-5Fluorouracil
   Bevacizumab 5mg/kg IV over 90 minutes Day 1
   Folinic acid 350mg IV over 2 hours Day 1
   Oxaliplatin 85mg/m² IV over 2 hours Day 1
   5Fluorouracil 400mg/m² IV bolus Day 1
   5Fluorouracil 2400mg/m² IV over 46 hours Day 1
   Repeated every 14 days6 cycles/3 months for 1st line metastatic colorectal cancer

3. Bevacizumab-Irinotecan-MdG
   Bevacizumab 5mg/kg IV over 90mins* Day 1 (max 675mg)
   Atropine 250mcg SC stat Day 1
   Irinotecan 180mg/m² IV over 30-90mins Day 1
   Folinic Acid 350mg IV over 2 hours Day 1
   5Fluorouracil 400mg/m² IV bolus Day 1
   5Fluorouracil 2400mg/m² IV over 46 hours Day 1
   Repeated every 14 days
4. **Bevacizumab-Cape-Irinotecan**  
   Bevacizumab 7.5mg/kg IV over 90mins* Day 1 (max 675mg)*  
   Atropine 250mcg SC stat Day 1  
   Irinotecan 200mg/m² IV over 30-90mins Day 1  
   Capecitabine 800mg/m² Oral twice a day Days 1 to 14, repeated every 21 days.

5. **Imatinib**  
   400mg Oral Once a day, continuous treatment, reviewed every 28 days  
   For Kit (CD117) positive, metastatic inoperable GIST without evidence of progression, continued until resistance develops

6. **Sorafenib**  
   Sorafenib 400mg Oral twice a day Continuous treatment  
   Consider starting at 200mg BD and escalating dose if no grade 2 or 3 toxicity  
   Blood tests repeated every 28 days.  
   1st line advanced stage Hepatocellular carcinoma or Advanced hepatocellular carcinoma, continued as long as clinical benefit or until unacceptable toxicity

7. **Sunitinib-50 (with break)**  
   Sunitinib 50mg Oral Once a day Days 1 to 28  
   Repeated every 42 days, i.e. 4 weeks treatment, followed by 2 week rest period.  
   Unresectable and/or metastatic malignant GIST if Imatinib treatment has failed because of resistance or intolerance and continued until disease progression.

8. **Sunitinib-37.5 Continuous**  
   Sunitinib 37.5mg Oral Once a day Days 1 to 28, repeated every 28 days, with no rest period. For unresectable or metastatic pancreatic neuroendocrine tumours (excluding poorly differentiated tumours) with disease progression, continued until progressive disease. For unresectable and/or metastatic malignant GIST if imatinib treatment has failed because of resistance or intolerance.
Embolisation Techniques

Yttrium 90 SIR – Spheres microspheres radio-embolisation plus OxMdG

**Cycle 1 (Full dose OxMdG)**
- Folinic acid 350mg IV over 2 hours Day 1
- Oxaliplatin 85mg/m² IV over 2 hours Day 1
- 5Fluorouracil 400mg/m² IV bolus dose Day 1
- 5Fluorouracil 2400mg/m² IV over 46 hours Day 1

**Cycle 2 only (Radio-embolism plus reduced dose OxMdG)**
- Folinic acid 350mg IV over 2 hours Day 1
- Oxaliplatin 60mg/m² IV over 2 hours Day 1
- 5Fluorouracil 400mg/m² IV bolus dose Day 1
- 5Fluorouracil 2400mg/m² IV over 46 hours Day 1
- Radio-embolism using SIR-spheres microspheres Day 3
  - Dose calculated based on body surface area (BSA), percentage tumour involvement and percentage lung shunting.

**Cycles 3 and 4 (Reduced dose OxMdG)**
- Folinic acid 350mg IV over 2 hours Day 1
- Oxaliplatin 60mg/m² IV over 2 hours Day 1
- 5Fluorouracil 400mg/m² IV bolus dose Day 1
- 5Fluorouracil 2400mg/m² IV over 46 hours Day 1

**Cycle 5 to 12**
- As cycle 1, repeated every 14 days as detailed above
- Only for loco-regional treatment of HCC confined to liver.
- Tests before starting course of chemotherapy include; preliminary arteriogram of liver (within 32 days of radio-embolisation) to determine vascular anatomy of the liver (to provide “road map” of arterial supply of liver to plan delivery of SIR-spheres,
macro-aggregated albumin (MAA) nuclear scan within 32 days of radio-embolisation to calculate the percentage of SIR-spheres that will pass through the liver and lodge in lungs due to arteriovenous shunts, Dose is adjusted to limit y99 damage to lungs, Contrast enhanced helical CT scan to calculate percentage tumour involvement (needed to calculate SIR-sphere dose).
## Appendix 6: Summary of tumour perfusion studies

<table>
<thead>
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</thead>
<tbody>
<tr>
<td><strong>Tumour group</strong></td>
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<tr>
<td>CRC with liver metastases</td>
<td>CRC</td>
<td>RCC</td>
<td>HCC</td>
<td>GIST</td>
<td>RCC</td>
<td>HCC</td>
<td>CRC</td>
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<tr>
<td><strong>Treatment</strong></td>
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<tr>
<td>Bevacizumab combination</td>
<td>Bevacizumab</td>
<td>Bevacizumab</td>
<td>Imatinib</td>
<td>Sunitinib</td>
<td>TACE</td>
<td>Sunitinib</td>
<td>Bevacizumab</td>
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<tr>
<td><strong>Sample size (patient number)</strong></td>
<td>6</td>
<td>30</td>
<td>42</td>
<td>30</td>
<td>21</td>
<td>3</td>
<td>30</td>
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<tr>
<td><strong>Machine settings</strong></td>
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</tr>
<tr>
<td>Philips iU22 (Bothell, WA, USA)</td>
<td>Aplio scanner (Toshiba Medical, Puteaux, France)</td>
<td>Aplio scanner (Toshiba Medical, Puteaux, France)</td>
<td>Aplio scanner (Toshiba Medical, Puteaux, France)</td>
<td>Aplio scanner (Toshiba Medical, Puteaux, France)</td>
<td>Philips IU22 (Philips Medical Systems, Seattle, WA, USA)</td>
<td>Philips IU22 (Philips Medical Systems, Seattle, WA, USA)</td>
<td>Aplio system Toshiba Medical system Europe, Germany</td>
</tr>
<tr>
<td>C5-2 mechanical index,0.06), 7-10 frames / second.</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>4.4 MHz Convex array low Mechanical Index (0.05 &lt; MI &lt; 0.2)</td>
<td>3.5 MHz Transducer</td>
</tr>
<tr>
<td>Loop length (minutes)</td>
<td>1 to 2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Breathing technique</td>
<td>No breath holding’ or ‘Deep breaths’</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Respirator motion iterative multiresolution registration algorithm Definity; Lantheus Medical Imaging, North Billerica, USA</td>
<td>Respirator motion iterative multiresolution registration algorithm Definity; Lantheus Medical Imaging, North Billerica, USA</td>
<td>Without any breath holding and respiratory gating technique</td>
</tr>
<tr>
<td>Respiratory gating technique applied</td>
<td>No breath holding’ or ‘Deep breaths’</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Normal breathing No respiratory compensatiion</td>
<td>Respirator motion iterative multiresolution registration algorithm Definity; Lantheus Medical Imaging, North Billerica, USA</td>
<td>Respirator motion iterative multiresolution registration algorithm Definity; Lantheus Medical Imaging, North Billerica, USA</td>
<td>Without any breath holding and respiratory gating technique</td>
</tr>
<tr>
<td>Contrast agent</td>
<td>SonoVue (Bracco, SPA, Milan Italy)</td>
<td>SonoVue (Bracco, SPA, Milan Italy)</td>
<td>SonoVue (Bracco, SPA, Milan Italy)</td>
<td>SonoVue (Bracco, SPA, Milan Italy)</td>
<td>Levovist and SonoVue (Bracco, SPA, Milan Italy)</td>
<td>Levovist and SonoVue (Bracco, SPA, Milan Italy)</td>
<td>SonoVue (Daiichi-Sankyo, Tokyo, Japan)</td>
</tr>
<tr>
<td>Dose</td>
<td>2.4 ml</td>
<td>4.8 ml</td>
<td>4.8 ml</td>
<td>4.8 ml</td>
<td>0.010 ml of encapsulated gas per kilogram of body weight</td>
<td>0.010 ml of encapsulated gas per kilogram of body weight</td>
<td>2.4 ml bolus followed by 10 ml normal saline flush</td>
</tr>
</tbody>
</table>
**Injection technique**
- Bolus -> 5 ml normal saline flush

**Quantification technique**
- QLAB Software (Philips medical system)
- Digitised quantification of contrast uptake
- Q Lab 8.1 (Philips medical system)
- Bracco QONTRAST software (Version 4.00)

**Schedule**
- Baseline + 2 weeks
- Baseline + 3 weeks
- Baseline + day 3
- Baseline + day 7 and 14
- Baseline + day 14
- Baseline + day 15
- Baseline + 2 weeks

**Findings**
- WITR, WIT 4/5 patients (80%) correct prediction at 2 weeks
- PI, TPI, AUC, AUWI, MTT at 2 weeks, significantly associate with RECIST at 2 months
- Perfusion parameters significantly associated with RECIST at 2 months
- Strong correlation (P<0.0001) between decrease in contrast uptake at day 7, 14 and tumour response
- Evidence of decrease in fractional blood volume of the tumour, however no correlation with RECIST at 2 months
- MTT quantitatively indicates early response to TACE in HCC patients
- TPI significantly (p<0.01) associated with RECIST at 2 weeks

**Limitations**
- Small sample size
- No prediction of PFS or OS
- Breathing compensation / respiratory gating and normalisation not used
- Breathing compensation / respiratory gating and normalisation not used
- Breathing compensation / respiratory gating and normalisation not used
- Breathing compensation / respiratory gating and normalisation not used
- Small sample size (3 patients) MTT only taken for 30 seconds
- Breathing compensation / respiratory gating technique not used

**Table: Tumour perfusion studies.** *TPI time to peak intensity * MTT mean transit time