Transcatheter hepatic therapy with irinotecan eluting beads (DEBIRI) for the treatment of colorectal liver metastases

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Robert Peter Jones

July 2013
Dedication

To Helen and my parents for their continued love, help and support. Thank you for giving me the opportunity to do this, as well as the motivation to complete it.
Acknowledgements

I owe an enormous amount to the many people who have helped me over the last few years.

**Dr. Neil Kitteringham**

The transition from clinician to scientist is notoriously difficult but the support, encouragement and seemingly endless patience demonstrated by you has made it (almost!) painless.

**Mr. Hassan Malik**

Your encouragement and enthusiasm is infectious. Without you, I doubt I would have achieved anywhere near as much as I have.

**Professor Graeme Poston**

For continuously inspiring me to aim higher than I’d ever thought of aiming. It’s very easy to make progress when someone like you is holding the door open.

**Mr. Stephen Fenwick**

Your time and experience were vital. Thank you for your help guiding me through the last few years.

**Professor Paula Ghaneh**

For your continued help and support, as well as your expert guidance on constructing a thesis.

I am also indebted to the numerous people who have helped me get to grips with working in the laboratory and the skills necessary to perform basic science. I hope I will be able to return the favour in the future.
Declaration

The work presented in this thesis was carried out in the Institute of Translational Medicine, University of Liverpool and was undertaken while working as a research fellow at the Northwest Hepatobiliary Unit, Aintree University Hospital.

RJ is a co-investigator in the PARAGON II trial. The trial is now complete, and formal analysis is underway by the industrial sponsor. The final results have not yet been approved for release. Discrepancies between the number of patients included in the trial, the translational assessment and radiological review reflect the relatively late approval for translational work as well as incomplete trial datasets at the time of submission.

I declare that all the work presented in this thesis has been carried out by me except where indicated below:

- Cation exchange prior to iTRAQ analysis was performed by Mrs. Jane Hamlett
- iTRAQ analysis was performed by Dr. Roz Jenkins
- Assessment of radiological response was performed by Dr. E. O’Grady and Dr. A. Smethurst
- Histopathological assessment of tumour response was performed by Dr. M. Terlizzo
Abstract

Background

There is growing interest in preoperative chemotherapy for patients with colorectal liver metastases (CRLM) but personalising treatments to maximise response and minimise toxicity remains a challenge. Transcatheter hepatic therapy with irinotecan-eluting beads (DEBIRI) allows targeted delivery of irinotecan direct to CRLM. However, the safety and efficacy of DEBIRI in a preoperative setting has not yet been defined. In addition, very little is understood about why response to DEBIRI varies between patients.

Aims

This thesis had 2 key aims: (1) To assess the safety and efficacy of neoadjuvant DEBIRI (2) To investigate inter-patient variations in treatment response.

Methods

Patients with resectable CRLM received a single treatment with DEBIRI 1 month prior to surgery (maximal dose 200mg). The primary end-point of the study was R0 tumour resectability. Hepatic parenchyma and CRLM were sampled at the time of resection. Hepatic expression of key metabolising enzymes was assessed using mass spectrometry based proteomics. Hepatic irinotecan metabolism was characterised and correlated with tumour response.

Results

DEBIRI was successfully administered in 40 patients. 1 patient (3%) developed post-DEBIRI pancreatitis. All 40 proceeded to surgery, with 38 undergoing resection. 30 day operative mortality was 5%, morbidity 27.5% (Clavien-Dindo 1-4). 63 discreet lesions were targeted, with 74% R0 resection rate. Histopathological examination found no residual tumour in 17% of lesions, <50% residual tumour in 59% and >50% tumour in 24%. 91% of treated lesions demonstrated stable disease by RECIST, with 9% demonstrating disease progression. RECIST was a poor predictor of pathological response or long-term outcome. At a median follow up of 293 days, fourteen patients (36%) had disease recurrence. On multivariate analysis, only tumour KRAS status was predictive of long-term outcome (p=0.02). There was a strong correlation between hepatic CES-2 expression and irinotecan activation (p < 0.001). Patients with a UGT1A1*28 6/7 SNP showed no difference in drug metabolism or pathological response. Hepatic CES-2 mediated activation of irinotecan clearly correlated with tumour replacement by fibrosis (p = 0.01).

Conclusions

This study demonstrates the safety and efficacy of neoadjuvant DEBIRI for CRLM, with impressive pathological response rates. Systemic exposure to irinotecan was low. The ability of hepatic tissue to activate irinotecan into SN-38 clearly correlated with tumour replacement by fibrotic tissue, suggesting that hepatic CES-2 activation is the key step in the effectiveness of DEBIRI. These preliminary data provide a pharmacological rationale for whole lobe embolisation and suggests a potential predictive biomarker of treatment efficacy for future validation.
Table of Contents

Dedication .................................................................................................................. 2
Acknowledgements .................................................................................................... 3
Abstract ...................................................................................................................... 5
List of Tables ............................................................................................................... 10
List of Figures ............................................................................................................. 12
List of Abbreviations ............................................................................................... 16

1 Introduction .............................................................................................................. 20
  1.1 Epidemiology of colorectal cancer ................................................................. 21
  1.2 Risk factors for colorectal cancer ................................................................... 22
     1.2.1 Dietary factors ............................................................................................... 22
     1.2.2 Lifestyle factors ............................................................................................. 22
     1.2.3 Inflammatory bowel disease ........................................................................ 23
  1.3 Aetiology of colorectal cancer ......................................................................... 23
     1.3.1 The adenoma–carcinoma sequence .............................................................. 23
  1.4 Molecular biology of colorectal cancer ........................................................... 24
     1.4.1 APC deactivation ......................................................................................... 25
     1.4.2 WNT-β catenin dysregulation ...................................................................... 26
     1.4.3 TGF-β dysregulation .................................................................................. 26
     1.4.4 KRAS/BRAF dysregulation .......................................................................... 27
     1.4.5 PI3K pathway dysregulation ....................................................................... 29
     1.4.6 p53 dysregulation ....................................................................................... 31
     1.4.7 Genomic and epigenomic colorectal cancer subtypes .............................. 31
        1.4.7.1 HNPCC .................................................................................................. 32
        1.4.7.2 Microsatellite instability (MSI) tumours .............................................. 32
        1.4.7.3 CpG island methylator phenotype (CIMP) and global DNA hypomethylation ............................................................... 33
        1.4.7.4 Chromosomal instability (CIN) tumours ........................................... 33
  1.5 Staging of colorectal cancer ............................................................................. 34
  1.6 Diagnosis of colorectal liver metastases ......................................................... 36
  1.7 Imaging of colorectal liver metastases ............................................................. 37
  1.8 Surgical management of colorectal liver metastases ...................................... 39
     1.8.1 Technical resectability ............................................................................... 40
     1.8.2 Oncological resectability .......................................................................... 41
     1.8.3 Techniques of surgical resection .................................................................. 43
     1.8.3.1 Transection techniques ......................................................................... 43
     1.8.3.2 Ablation for colorectal liver metastases .............................................. 44
     1.8.3.3 Laparoscopic liver surgery ..................................................................... 45
  1.9 Chemotherapeutic management of colorectal liver metastases .................. 46
     1.9.1 Chemotherapy for resectable colorectal liver metastases ....................... 47
     1.9.1.1 Adjuvant therapy for resectable colorectal liver metastases ............ 48
     1.9.1.2 Neoadjuvant therapy for resectable colorectal liver metastases .... 49
     1.9.2 Chemotherapy for irresectable disease ..................................................... 52
     1.9.3 Chemotherapy-induced liver injury ............................................................ 58
     1.9.4 Radiological assessment of chemotherapeutic response ....................... 60
     1.9.5 Pathological assessment of chemotherapeutic response ....................... 62
  1.10 Prognostic and predictive markers for colorectal liver metastases .............. 64
     1.10.1 Clinicopathological staging as a predictor of outcome ......................... 64
1.11 Locoregional management of colorectal liver metastases.......... 73
  1.11.1 Hepatic arterial infusion............................................ 73
  1.11.2 Trans-arterial chemoembolisation (TACE).......................... 74
  1.11.3 Drug eluting beads for TACE (DEB-TACE).......................... 76
  1.11.3.1 Animal and preclinical assessment of DEBIRI-TACE in metastatic liver disease.................................................. 78
  1.11.3.2 Clinical assessment of DEBIRI-TACE in irresectable CRLM ...... 80
  1.11.3.3 Pharmacokinetics of irinotecan after DEBIRI-TACE.......... 83
1.12 Irinotecan ................................................................................. 84
  1.12.1 Topoisomerase 1 ............................................................. 85
  1.12.2 Metabolism of irinotecan ................................................... 86
    1.12.2.1 Carboxylesterase mediated hydrolysis.......................... 87
    1.12.2.2 CYP3A metabolism ................................................... 89
    1.12.2.3 UDP-glucuronosyltransferase....................................... 91
    1.12.2.4 Adenosine-triphosphate binding cassette (ABC) transporters... 92
    1.12.2.5 β-glucuronidase biotransformation............................... 93
  1.12.3 Transport of irinotecan in blood.......................................... 94
  1.12.4 Pharmacokinetics after intravascular administration of irinotecan .... 95
  1.12.5 Enterohepatic reactivation of irinotecan............................... 95
  1.12.6 Metabolism of irinotecan in patients with hepatic disease .......... 96
1.13 Aim of thesis............................................................................. 97
1.14 Plan of study................................................................. 97
2 PARAGON II trial of neoadjuvant DEBIRI for colorectal liver metastases. 99
  2.1 Introduction ............................................................... 100
  2.2 Methods ................................................................. 101
    2.2.1 Study Design ......................................................... 101
    2.2.2 Patient identification ................................................ 101
    2.2.3 Inclusion criteria .................................................... 101
    2.2.4 Exclusion criteria .................................................... 102
    2.2.5 Study endpoints ...................................................... 102
      2.2.5.1 Primary endpoint ................................................. 102
      2.2.5.2 Secondary endpoints ............................................ 103
    2.2.6 Study schedule ....................................................... 107
      2.2.6.1 Baseline assessment .............................................. 107
      2.2.6.2 Neoadjuvant chemoembolisation ................................ 108
      2.2.6.3 Surgery ............................................................ 109
      2.2.6.4 Follow-up visits ................................................ 110
      2.2.6.5 Statistical analysis ............................................. 110
  2.3 Results ............................................................................. 112
    2.3.1 Patient demographics .................................................. 112
    2.3.2 DEBIRI-TACE .......................................................... 113
    2.3.3 Surgery .................................................................... 113
    2.3.4 Pathological response .................................................. 114
      2.3.4.1 Steatosis and steatohepatitis after DEBIRI-TACE .......... 116
      2.3.4.2 Comparison of patient characteristics by pathological response rate 117
    2.3.4.3 Comparison of treated and untreated lesions in the same patient119
    2.3.5 Radiological response ................................................... 122
      2.3.5.1 Radiological response and pathological tumour response ...... 122
    2.3.6 Long-term outcome ...................................................... 126
      2.3.6.1 Overall survival .................................................. 126
4.2.7.2 Targeted proteomic analysis using Western immunoblotting ...... 182
4.2.8 UGT1A1 genotyping ................................................................. 184
  4.2.8.1 DNA extraction ................................................................. 184
  4.2.8.2 Primer development .......................................................... 184
  4.2.8.3 PCR conditions ............................................................... 185
  4.2.8.4 Analysis of PCR products .................................................. 185
4.2.9 Statistical analysis ............................................................... 186
4.3 Results .................................................................................. 187
  4.3.1 Biosampling ........................................................................ 187
  4.3.2 Pathological response rates .................................................. 188
  4.3.3 Targeted and global proteomic analysis .................................. 188
    4.3.3.1 iTRAQ quantification ...................................................... 188
    4.3.3.2 Western blotting ............................................................ 189
    4.3.3.3 Comparison of protein quantification between iTRAQ and Western immunoblotting ....................................................... 191
  4.3.4 UGT1A1 genotyping ............................................................ 193
    4.3.4.1 Agarose gel visualisation ............................................... 193
    4.3.4.2 Agilent 1200 bioanalysis ............................................... 193
    4.3.4.3 Effect of UGT1A1*28 SNP on protein expression .............. 195
  4.3.5 Serum metabolic profiling .................................................... 195
    4.3.5.1 Serum concentrations of drug and metabolites ................. 195
    4.3.5.2 Hepatic parenchymal protein expression and serum drug concentrations .......................................................... 200
  4.3.6 Microsomal metabolism of irinotecan .................................... 202
    4.3.6.1 Microsomal metabolite production .................................. 202
    4.3.6.2 Hepatic parenchymal expression and microsomal drug production 204
  4.3.7 Correlation between metabolic profile and pathological tumour response 206
    4.3.7.1 Serum concentration and pathological response ............... 206
    4.3.7.2 Protein expression and pathological response ................... 206
    4.3.7.3 Microsomal production and pathological response ............ 207
4.4 Discussion ............................................................................. 209
5 Concluding Discussion ................................................................ 213
Bibliography .............................................................................. 226
Appendix 1 – Supporting publications & presentations .................... 273
Appendix 2 - Preclinical data assessing irinotecan pharmokinetics after treatment with DEBIRI .......................................................... 276
Appendix 3 – Research Ethics Approval ........................................... 281
List of Tables

Table 1 UICC TNM Staging of Colorectal Cancer ........................................ 35

Table 2 UICC/AJCC Stage grouping of TNM staging. .............................. 36

Table 3 Table showing response rate and resection rate for key trials of cytotoxic agents with the addition of targeted biological agents in patients with initially irresectable metastatic colorectal cancer ........................................ 54

Table 4 Comparison of Nordlinger and Fong Clinical Risk Score. ............ 67

Table 5 The Basingstoke Prognostic Index ............................................. 69

Table 6 Prognostic biomarkers in colorectal cancer ............................... 71

Table 7 Predictive biomarkers in colorectal cancer ................................. 72

Table 8 Summary of Kleiner score .......................................................... 106

Table 9 Demographic details of patients undergoing treatment with DEBIRI ....... 112

Table 10 Characteristics of patients who underwent hepatectomy for CRLM after neoadjuvant DEBIRI stratified by pathological tumour response .................. 118

Table 11 Demographics of patients assessed for radiological response .......... 122

Table 12 Univariate and multivariate analysis of clinicopathological variables associated with DFS in patients treated with DEBIRI .............................. 128

Table 13 Source, purity and molecular mass of reference analytes used for assay development ................................................................. 141

Table 14 Amount of analyte on column for each concentration of biosample .... 144

Table 15 Ion source and analyte dependent MS parameters ....................... 149

Table 16 Accuracy and precision analysis of human liver microsomes and serum spiked with 4 analytes at LLOQ .................................................. 152

Table 17 Inter-batch accuracy and precision for three microsomal QC samples at LLOQ, mid and high range. ...................................................... 155
Table 18 Inter-batch accuracy and precision for three serum QC samples at LLOQ, mid and high range. ................................................................. 155

Table 19 Intra-batch accuracy and precision for 3 microsomal QC samples at LLOQ, mid and high range. ................................................................. 157

Table 20 Intra-batch accuracy and precision for 3 serum QC samples at LLOQ, mid and high range. ........................................................................ 157

Table 21 Analyte recovery from microsomal extract & serum ............................................. 158

Table 22 Amount of analyte detected in 100nM standard protein-precipitated matrix stored at room temperature over time ........................................... 159

Table 23 Amount of analyte detected in 100 nM standard after three freeze-thaw cycles ................................................................................................. 159

Table 24 Primary antibodies used for Western blotting ................................................ 183

Table 25 Content of PCR incubation samples .............................................................. 185

Table 26 Biosampling record sheet for patients recruited into translational arm of PARAGON II study ................................................................. 187

Table 27 Tumour pathological response rate after neoadjuvant treatment with DEBIRI-TACE ................................................................. 188

Table 28 Parenchymal protein expression by iTRAQ ........................................ 189

Table 29 Microsomal protein expression by Western immunoblotting ...................... 191

Table 30 Serum drug concentrations 1- and 6-hours post-DEBIRI ......................... 197

Table 31 Microsomal drug metabolism ....................................................................... 202

Table 32 Pathological response rates reported after neoadjuvant treatment with systemic chemotherapy ................................................................. 216
List of Figures

Figure 1 Downstream mediators of epidermal growth factor (EGFR) signaling ...... 28

Figure 2 Proposed genetic model of adenoma-carcinoma sequence................. 30

Figure 3 Kaplan-Meier survival curves from EORTC 40983 trial comparing perioperative chemotherapy vs. resection alone for patients with initially resectable CRLM............................................ 52

Figure 4 Kaplan-Meier survival curves showing survival following hepatectomy for CRLM comparing those who were initially resectable at presentation to those patients who were considered initially unresectable but were brought to resection using systemic chemotherapy........ Error! Bookmark not defined.

Figure 5 Rates of secondary liver resection following systemic chemotherapy.............................................................................. 55

Figure 6 Representative photomicrographs of chemotherapy associated liver injury ........................................................................................................ 59

Figure 7 Kaplan-Meier survival curves of patients treated with systemic neoadjuvant chemotherapy stratified by pathological response grade................. 63

Figure 8 Kaplan–Meier survival curves from a population of 110 000 UK patients diagnosed with primary colorectal cancer stratified by disease stage......... 64

Figure 9 Kaplan-Meier plot showing observed cancer-specific survival after primary liver resection for colorectal liver metastases stratified across 5 groups of increasing score on the Basingstoke Prognostic Index......................... 68

Figure 10 Preclinical analysis of serum drug and metabolite concentrations after treatment with DEBIRI........................................................................ 79

Figure 11 Schematic diagram showing intracellular metabolism of irinotecan........ 2

Figure 12 Tumour sampling strategy................................................................. 105

Figure 13 Representative photomicrographs of pathological tumour response after treatment with DEBIRI................................................................. 115

Figure 14 Column chart showing pathological response grade of colorectal liver metastases treated with DEBIRI................................................................. 115
Figure 15 Column chart showing Kleiner score for steatosis, steatohepatitis and fibrosis in background hepatic parenchyma from patients treated with DEBIRI.

Figure 16 Photomicrograph (x20) of (A) targeted and (B) non-targeted CRLM from the same patient treated with DEBIRI.

Figure 17 CT scan of patients [A] before and [B] 34 days after single treatment with DEBIRI.

Figure 18 Box and whisker plot showing correlation between morphological response and pathological tumour assessment.

Figure 19 Kaplan-Meier curve showing disease free survival after hepatic resection following neoadjuvant DEBIRI-TACE.

Figure 20 Kaplan-Meier curve showing the negative prognostic effect of KRAS mutation on disease free survival after neoadjuvant treatment with DEBIRI-TACE.

Figure 21 Solvent gradient used for HPLC separation of analytes.

Figure 22 Representative chromatogram of 1.5 pmol of on column analyte extracted from microsomal fraction.

Figure 23 ESI mass spectra for (A) irinotecan, (B) SN-38, (C) APC and (D) camptothecin showing parent and product ion.

Figure 24 Selectivity shown by representative chromatograms obtained from (a) blank microsomal fraction and (b) drug-free serum, demonstrating assay specificity.

Figure 25 Representative standard curves obtained for irinotecan and its metabolites.

Figure 26 Representative chromatograph of analytes spiked and then extracted from microsomal matrix at LLOQ.

Figure 27 LC-MS/MS chromatograph of a representative biological sample.

Figure 28 Graph showing SN-38 production at increasing concentrations of irinotecan.
Figure 29 Graph showing increasing SN-38 production with increasing protein concentration.................................................................................................................. 162

Figure 30 Graph showing linear production of SN-38 with increasing protein concentration.................................................................................................................. 162

Figure 31 Graph showing SN-38 production in the presence of the cofactors UDPGA & NADPH.................................................................................................................. 162

Figure 32 Graph showing SN-38G production with the addition of UDPGA................................................................................................................................. 163

Figure 33 Graph showing SN-38 production in the presence of the cofactors UDPGA & NADPH.................................................................................................................. 163

Figure 34 Graph showing SN-38 production irrespective of the addition of enzymatic cofactors.................................................................................................................. 164

Figure 35 Graph showing APC production with the addition of NADPH ............. 164

Figure 36 Graph showing APC production with the addition of NADPH ............. 165

Figure 37 Graph showing SN-38G formation (AUC) increases with increasing concentrations of SN-38.................................................................................................................. 166

Figure 38 Graph showing the formation of SN-38G in human liver microsome incubated with UDPGA.................................................................................................................. 166

Figure 39 Graph showing the formation of SN-38G in human liver microsome incubated with UDPGA.................................................................................................................. 167

Figure 40 Standard curve used for the quantification of SN-38G in all incubations. .................................................................................................................................................. 167

Figure 41 Standard curve used for the quantification of SN-38G in all incubations. .................................................................................................................................................. 168

Figure 42 Representative photomicrographs of colorectal liver metastases treated with DEBIRI showing differing pathological responses .............................................. 168

Figure 43 Representative western blot and densitometry of patients parenchyma blotted for key proteins.................................................................................................................. 168

Figure 44 Scatter plot showing comparison of protein quantification using Western immunoblotting and iTRAQ.................................................................................................................. 170
Figure 45  Agarose gel stained with ethyl bromide to visualize products of UGT1A1*28 PCR.

Figure 46  Microcapillary gel from Agilent Bioanalyser 2100 of showing products of UGT1A1*28 PCR.

Figure 47  Representative electropherograms for UGT1A1*28 PCR products.

Figure 48  Composite graphs showing serum concentrations of (A) irinotecan, (B) SN-38, (C) SN-38G and (D) APC for 8 patients at 0, 1 and 6 hours post-DEBIRI.

Figure 49  Scatter plot showing correlation between dose of DEBIRI and serum concentrations of irinotecan at 1 and 6 hours.

Figure 50  Column chart showing ratio of SN38:irinotecan.

Figure 51  Box and whisker plot of serum and drug metabolism quantified by iTRAQ expression.

Figure 52  Column chart showing amount of metabolite present after microsomal incubation of 500 nM irinotecan for 120 minutes.

Figure 53  Scatter plot showing relationship between SN38 and SN38-G.

Figure 54  Scatter plot showing the correlation between microsomal drug metabolism and protein expression.

Figure 55  Scatter plot showing relationship between hepatic parenchymal expression of CES-2 and tumour fibrosis after neoadjuvant DEBIRI-TACE.

Figure 56  Scatter plots showing correlation between microsomal SN-38 and validated pathological markers of tumour response.

Figure 57  CONSORT diagram for proposed PARAGON III RCT.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td></td>
<td>Also</td>
</tr>
<tr>
<td></td>
<td>7-ethyl-10-aminopentanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>CALI</td>
<td>Chemotherapy associated liver injury</td>
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<tr>
<td>CAPOX</td>
<td>Capecitabine/Oxaliplatin</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CEP</td>
<td>Collision excitation potential</td>
</tr>
<tr>
<td>CES</td>
<td>Carboxylesterase</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG island methylator phenotype</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum concentration</td>
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<tr>
<td>CR</td>
<td>Radiological complete response</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>CRLM</td>
<td>Colorectal liver metastases</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>cTACE</td>
<td>Conventional transarterial chemoembolisation</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>CX</td>
<td>Collision energy</td>
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<tr>
<td>CYP3A4</td>
<td>Cytochrome p450 3A4</td>
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<tr>
<td>DEBIRI</td>
<td>Drug eluting beads with irinotecan</td>
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<tr>
<td>DFS</td>
<td>Disease free survival</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Declustering potential</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
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<td>FUDR</td>
<td>Floxuridine</td>
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<tr>
<td>HAI</td>
<td>Hepatic arterial infusion</td>
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<tr>
<td>HNPCC</td>
<td>Hereditary non polyposis colorectal cancer</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IOUS</td>
<td>Intraoperative ultrasound scan</td>
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<tr>
<td>iTRAQ</td>
<td>Isobaric Tag for Relative and Absolute Quantification</td>
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<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistance transporter</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>mRECIST</td>
<td>Modified Response Evaluation Criteria in Solid Tumours</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MSI-H</td>
<td>Microsatellite instability high</td>
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<tr>
<td>MSI-L</td>
<td>Microsatellite instability low</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NICE</td>
<td>UK National Institute for Clinical Excellence</td>
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<tr>
<td>NICE</td>
<td>UK National Institute for Healthcare and Clinical Excellence</td>
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<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PR</td>
<td>Radiological partial response</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumours</td>
</tr>
<tr>
<td>SD</td>
<td>Radiological stable disease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TACE</td>
<td>Trans arterial chemoembolisation</td>
</tr>
<tr>
<td>TEAB</td>
<td>Triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time to maximum concentration</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td>TWEEN</td>
<td>Polyoxyethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>UDPGA</td>
<td>Uridine 5'-diphospho-glucuronic acid</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>UDP-glucuronosyltransferase 1-1</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>XELOX</td>
<td>Capecitabine/Oxaliplatin</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>Microgramme</td>
</tr>
<tr>
<td>$\mu l$</td>
<td>Microlitre</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>Micromole</td>
</tr>
<tr>
<td>$\mu m$</td>
<td>Micrometre</td>
</tr>
</tbody>
</table>
1 Introduction
1.1 Epidemiology of colorectal cancer

Colorectal cancer is the fourth commonest cancer in the UK, with 40,000 new cases diagnosed every year. It is the third highest cause of cancer-related death in the UK, with 16,259 people dying from the disease in 2012. Prognosis is improving, with survival rates climbing dramatically. For those diagnosed between 1971-75, 5-year survival was 25% for men and 28% for women compared to 50% and 51% for those diagnosed between 2001-2006 (Cancer Research UK, 2012). Population level studies have highlighted the increasing prevalence of colorectal cancer, possibly because of an aging population. Incidence is directly associated with increasing age; 71% of new cases occur in patients over 65 years, and 42% occur in those older than 75 years (Eheman 2012). According to US Surveillance, Epidemiology and End Results (SEER) and Census Bureau data, an estimated increase in CRC incidence of 52% is projected over the next 20 years (Smith et al., 2009).

Stage specific survival varies, with over 93% of patients diagnosed with Dukes A colorectal cancer surviving 5 years, compared to less than 5% for those with metastatic disease. There is also a survival difference between social groups within the UK, with the most affluent having a 5-9% increased 5-year survival compared to the least well off (Coleman et al., 2004). Perhaps unsurprisingly, the incidence of colorectal cancer is not uniform across the UK with the highest incidence in deprived urban areas (Dunlop, 1992) - a trend reflected across Europe (Bray et al., 2002).
1.2 Risk factors for colorectal cancer

1.2.1 Dietary factors

Several studies have suggested a link between a diet low in fruit and vegetables and colorectal cancer (Terry et al., 2001; Reedy et al., 2008) although hypotheses vary as to whether it is the fibre, antioxidant or flavone content of these foodstuffs that exerts this protective effect. The evidence for fibre exerting a protective effect is reinforced by several large observational studies showing an inverse correlation between fibre intake and colorectal adenomas and carcinomas (Negri et al., 1998; Peters et al., 2003; Bingham et al., 2003). However, a 2002 Cochrane Review suggested there was insufficient evidence to support increasing fibre intake to reduce the incidence of colorectal adenoma (Asano et al., 2002). It has also been suggested that red meat and, indirectly, high saturated fat intake may be related to the incidence of colorectal cancer (Sandhu et al., 2001). An association between excess alcohol intake and colorectal cancer has been supported by meta-analysis (Longnecker et al., 1990) and it has been suggested that this may be related to reduced folate intake and absorption (Harnack et al., 2002).

1.2.2 Lifestyle factors

Observational studies have suggested that low levels of exercise are associated with increased risk of developing colorectal cancer. However, this effect may be confounded by higher levels of obesity, metabolic syndrome and diabetes amongst patients with low levels of activity (Mao et al., 2003). Smoking is also associated with an increased risk of colorectal cancer (Colangelo et al., 2004).
1.2.3 Inflammatory bowel disease

Patients with inflammatory bowel disease such as Ulcerative Colitis and Crohn’s disease are at significantly increased risk for colorectal cancer, presumably as inflammatory changes trigger dysplasia (Choi et al., 1994). In patients with Ulcerative Colitis, risk seems to be highest in those with pancolitis and appears to rise markedly after 10 years of symptoms (Gyde et al., 1988) with a cumulative incidence of 5-10% after 20 years, 12-20% at 30 years and 30% at 40 years (Ekbom et al., 1990). There is growing evidence that the risk of colorectal cancer in longstanding Crohn’s disease is similar to that of Ulcerative Colitis (Ekbom et al., 1990). As might be expected, the mean age of incidence for inflammatory bowel disease-related colorectal cancer is lower than for sporadic cases (45 vs. 60 years) (Choi et al., 1994).

1.3 Aetiology of colorectal cancer

1.3.1 The adenoma-carcinoma sequence

Localized lesions within the colon that project above the mucosa are commonly termed polyps. Most colorectal polyps, particularly those smaller than 5 mm in size, are hyperplastic (a large number of normal appearing cells) and are not thought to develop into cancer. Adenomas arise from glandular epithelium within the lining of the large bowel and have distorted morphology, with altered appearance of the epithelial cells, and premalignant potential (Jass, 2007). Around 50% of patients aged 70 will have at least one adenoma (Rex et al., 1993). Polypectomy reduces the risk of colorectal cancer, and patients who have their polyps removed at endoscopy have a lower incidence of malignant disease (Winawer et al., 1993). Adenocarcinoma can often be detected in adenomatous polyps, and regions of
adenomatous epithelium are often observed in resected colorectal cancer specimens (Fearon, 2011). It is thought that only a small proportion of adenomas progress to cancer; adenomas approximately 1 cm in size have a 10% to 15% chance of progressing to carcinoma over a 10-year period (Stryker et al., 1987).

These findings have led to the development of the adenoma-carcinoma concept, first hypothesized by Fearon and Vogelstein (Fearon et al., 1990). This hypothesis was developed following a number of observations. The incidence of adenomatous polyps is highest in the distal colon, where there is also the highest incidence of colorectal cancer. Clark et al demonstrated that geographical locations with high incidence of colorectal cancer were the same regions with high incidence of adenomatous polyps (Clark et al., 1985). Age-distribution curves for adenomas and carcinomas also show an increase with age, although incidence of adenomas peaks 5 years before that of carcinomas (Muto et al., 1975).

1.4 Molecular biology of colorectal cancer

Most patients with colorectal cancer have no identifiable hereditary risk factors. However, around 20% occur on a background of an immediate family member having a primary colorectal malignancy, implying some degree of inherited predisposition (Lynch et al., 2003). A smaller proportion (approximately 10%) occurs in patients with recognized genetic syndromes that predispose towards gastrointestinal malignancy, such as hereditary non-polyposis colorectal cancer (HNPCC) (see section 1.4.7.1) or familial adenomatous polyposis (FAP). The remaining 80% of colorectal cancers are considered sporadic in nature, with the cancer developing from multi-potent stem cells located within intestinal crypts. Extensive work on the molecular pathogenesis of colorectal cancer suggests that
the majority of genetic alterations in colonic epithelial cells are harmless, triggering no functional change in phenotype. However, a number of key genomic and epigenomic changes have been identified which give rise to functional change and are positively selected for during carcinogenesis (Wood et al., 2007). These mutations tend to be intrinsically involved in DNA stability and repair, migration, cell adhesion and proliferation and lead to the development of adenomas and carcinomas.

1.4.1 APC deactivation

In FAP, multiple colonic adenomas occur throughout the length of the colon in the late teens and early twenties. The large number and early incidence of adenomas results in the formation of carcinomas in most patients by age 50 (Lynch et al., 2003). The number of polyps makes surveillance endoscopy impossible, and most patients undergo prophylactic subtotal colectomy with surveillance of the rectal stump. The condition is caused by mutation of the adenomatous polyposis coli (APC) gene, with truncating germ-line mutations the cause of classical APC (Lynch et al., 2003). The APC tumour suppressor gene encodes a 300-kDa protein that integrally regulates cell-to-cell adhesion, migration, chromosomal segregation, and apoptosis (Fearon et al., 1990).

As well as being integrally involved in the development of FAP, APC is also fundamental to the development of sporadic colorectal cancer; around 75% of sporadic tumours have somatic mutations that inactivate APC. There is good evidence that somatic APC mutations are an early and potentially rate-limiting step in the development of the adenoma-carcinoma sequence. APC mutations occur with the same frequency in very small adenomas, advanced adenomas and carcinomas (Fearon et al., 1990) and APC mutations are found in all adenomas, even microscopic adenomas with only a small number of dysplastic glands (Kinzler
et al., 1996). The most established role for APC in the development of cancer is as a major binding partner and regulator of the β-catenin/WNT signaling pathway.

1.4.2 WNT- β catenin dysregulation

Mutations in the APC/WNT-β-catenin signaling pathway have been reported in 98% of colorectal cancers (Cancer Genome Atlas Network, 2012). APC down regulates WNT signaling by targeting β-catenin for degradation. Inactivation of APC leads to nuclear β-catenin accumulation, increased WNT signaling and cellular proliferation. Despite the clear role of WNT-signal dysregulation in the development of colorectal cancer, targeted agents tackling this mutation have yet to move beyond preclinical testing (Pritchard et al., 2011).

1.4.3 TGF- β dysregulation

TGF-β signaling is dysregulated in the majority of colorectal cancers, and mutations have been identified in genes coding for extracellular receptors (TGFBR1, TGFBR2), postreceptor signaling genes (SMAD2, SMAD4) and superfamily genes (ACVR2) (Pritchard et al., 2011).

Mutations in TGFBR2 are detected in around 30% of colorectal cancers, and have been associated with malignant transformation in late adenomas (Grady et al., 1999). SMAD4 is located on 18q, a region commonly deleted in colorectal cancer. Loss of this gene is associated with progression through the adenoma-carcinoma sequence in mouse models, supporting its role as a tumour suppressor gene (Taketo et al., 2000). Furthermore, loss of SMAD4 has been suggested as predictor of a more aggressive cancer phenotype and is associated with local invasion and lymph node metastases (Tanaka et al., 2008).
1.4.4 KRAS/BRAF dysregulation

KRAS is a proto-oncogene, and is arguably the most clinically important oncogene in the development of colorectal cancer. KRAS is a downstream effector of endothelial growth factor receptor (EGFR), with binding of the ligand to the receptor triggering downstream signaling via the PI3K/AKT/MTOR and RAF/MEK/ERK proliferation pathways (Figure 1) (Fearon, 2011). Mutations in codon 12, 13 and less frequently codon 61 of KRAS have been reported in approximately 40% of colorectal tumours (Downward, 2003) with almost perfect concordance between primary and metastatic tumour (Artale et al., 2008; Vakiani et al., 2012). This concordance is exploited clinically as decisions on the appropriateness of treatment with targeted EGFR antibodies in a metastatic setting are based on primary tumour KRAS status. Patients with KRAS mutant tumours have previously been shown to derive no benefit from the administration of the targeted antibodies Cetuximab and Panitumumab (see section 1.9). However, this treatment algorithm is now being questioned with growing evidence that patients with KRAS G13-D mutant tumours may benefit from Cetuximab alongside cytotoxic chemotherapy (Tejpar et al., 2012). To date, KRAS remains the only clinically utilized predictive biomarker to guide therapeutic choice.
Figure 1 Downstream mediators of epidermal growth factor (EGFR) signaling. EGFR stimulation leads to activation of the KRAS/BRAF/MAPK pathway and indirectly the PI3K/AKT pathway, triggering cell growth, differentiation, survival and invasion. Monoclonal antibodies such as Cetuximab and Panitumumab act by blocking EGFR receptor stimulation. Downstream mutations in RAS/RAF/MAPK (leading to constitutive activation of KRAS/BRAF) pathways can render these agents ineffective. Adapted from Pritchard et al., 2011.

The BRAF gene codes for a protein kinase downstream of KRAS in the RAS/RAF/MEK/ERK pathway. The overwhelming majority of BRAF mutations are single base changes resulting in the substitution of glutamic acid for valine at codon 600 (V600E) (Siena et al., 2009). BRAF and KRAS mutations appear to be mutually exclusive, supporting the hypothesis that only one mutation in this pathway is required to trigger upregulation of MAPK signaling (Rajagopalan et al., 2002). Interestingly, BRAF mutation in colorectal cancer has been associated with global
DNA hypermethylation (CIMP-high, see section 1.4.7) (Ogino et al., 2008). High levels of DNA methylation are associated with increased sensitivity to Camptothecin (Orta et al., 2009) and it has been hypothesized that hypermethylated BRAF-mutated tumours may be more sensitive to irinotecan. This was supported by the retrospective analysis of BRAF status in patients treated as part of the large CALGB 89803 RCT comparing 5-FU/LV vs. 5-FU/LV/irinotecan in patients with stage III CRC which demonstrated a non-significant trend towards improved OS for BRAF-mutant patients treated in the irinotecan-containing arm (HR 0.52, 95% CI 0.25-1.10) (Ogino et al., 2012). By contrast, the UK MRC FOCUS trial performed a retrospective analysis on 711 patients with stage IV CRC treated with a combination of non-targeted therapies (5-FU+/−Oxaliplatin/irinotecan). Mutation in KRAS (Codon 12, 13, 61) or BRAF (V600E) was associated with a poor overall survival (HR, 1.40; 95% CI, 1.20 to 1.65; P < .0001) but had minimal impact on progression-free survival (HR, 1.16; 95% CI, 1.00 to 1.36; P = .05) irrespective of treatment (Richman et al., 2009).

1.4.5 PI3K pathway dysregulation

The PI3K pathway is most commonly mutated in the p110α subunit of PI3KCα and is thought to promote the transition from adenoma to carcinoma (Figure 2) (Samuels et al., 2004). The PI3K pathway is modulated in part by KRAS, and it has been postulated that both PI3K and KRAS status may predict outcome after anti-EGFR treatment (Razis et al., 2008) although this has yet to be validated in a clinical setting.
Figure 2 Proposed genetic model of adenoma-carcinoma sequence (a)

Sporadic mutations accumulate, leading to development of malignant phenotype. The order in which mutations are accumulated is unimportant, although it has been suggested that certain mutations (e.g. APC, KRAS) are associated with early adenoma formation. (b) In some cancers, MMR function is inactivated either by somatic mutations (HNPCC) or by epigenetic inactivation (CIMP), leading to high-frequency microsatellite instability (MSI-H). These epigenetic changes then lead to tumour-suppressor inactivation via microsatellite instability or promoter hypermethylation. Adapted from Fearon, 2011.
1.4.6 p53 dysregulation

p53 protein is a key transcriptional regulator for numerous cell-cycle checkpoints, promotes apoptosis in damaged cells and restricts angiogenesis (Vousden et al., 2009). The high prevalence of p53 mutations in colorectal cancer suggests that stresses on tumour cells (the frequent need to activate cell-cycle arrest, apoptosis etc.) may apply a selective pressure in favour of a clonal subtype with p53 deficiency, leading to continued growth and the acquisition of invasive properties. Baker et al. (Baker et al., 1990) assessed p53 status in 58 resected colorectal tumours. Isolated p53 mutations were rarely seen in carcinomas that contained both copies of chromosome 17p (17%). However, in 70% of tumours that contained 17p allelic deletions (the location of the p53 gene) the remaining p53 gene was mutated over 80% of the time. The authors concluded that a single point mutation is the rate limiting step and once a single mutated allele has developed, p53 deactivation rapidly follows.

1.4.7 Genomic and epigenomic colorectal cancer subtypes

As well as interest in the full DNA coding sequence of colorectal cancer (genomics), there is a growing recognition that modifications to DNA molecules that do not directly affect the DNA coding sequence can regulate gene activity (epigenomics). Epigenomics can explain whether genes are activated, and can influence protein function within cells. Importantly, epigenomic changes can be passed from generation to generation, and can also be affected by a lifestyle factors – for example, diet and exposure to pollutants. A number of distinct genomic and epigenomic colorectal cancer phenotypes have been recognized.
1.4.7.1 HNPCC

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant inherited condition that leads to a high risk of cancer, especially colorectal cancer (25-70% lifetime risk) (Vasen et al., 2013). This increased risk is due to patients inheriting germ-line mutations in mismatch repair (MMR) genes which repair DNA replication errors. Classically, multiple generations of a family are affected with a much earlier mean age of incidence than sporadic cases. Over 70% of these lesions are right sided, and are found proximal to the splenic flexure. Unsurprisingly, these patients have a higher than average number of synchronous lesions (Lynch et al., 1999). Patients with HNPCC undergo enhanced carcinogenesis, with progression from adenoma to carcinoma within 2-3 years, as opposed to 8-10 years in general population (Lynch et al., 1999). Sufferers of HNPCC are also at risk of developing other primary tumours, including stomach, small bowel, pancreas and hepatobiliary malignancies (Aarnio et al., 1999). As understanding of the molecular biology of colorectal cancer grew, investigators sought to identify the specific loss of function mutation in these affected individuals. These studies failed to identify a relevant sequence, but found the microsatellite DNA sequences used for the analysis showed marked variations in length compared to normal tissue from the same patient (Aaltonen et al., 1993). The phenotype was ultimately termed the microsatellite instability (MSI) phenotype (See section 1.4.7.2).

1.4.7.2 Microsatellite instability (MSI) tumours

Microsatellite instability is a condition of genetic hypermutability that results from impaired mismatch repair mechanisms, and is detected in approximately 15% of colorectal cancers. Tumours are defined as high-MSI or low-MSI based on the proportion of unstable loci in a panel of 5-10 points (> or < 30%) (Boland et al., 1998). Patients with MSI tumour types have been shown to have a favourable
prognosis compared to patients with chromosomal instability tumours (CIN) (Popat et al., 2005), and it has been suggested that these tumour types may respond differently to chemotherapeutic agents (Jo et al., 2006). MSI is thought to be due to inactivation of genes integral to DNA repair - the DNA mismatch repair (MMR) family, which includes MLH1, MSH2, MSH6 and PMS2 (Grady et al., 2008). During cancer development, cells with impaired DNA repair manifest a mutator phenotype and accumulate mutations in a much more rapid fashion than cells with functioning DNA repair function (Aaltonen et al., 1993).

1.4.7.3 CpG island methylator phenotype (CIMP) and global DNA hypomethylation
Epigenetic instability in colorectal cancer is associated with both global DNA hypomethylation, as well as hypermethylation of CpG islands. CIMP is defined as methylation of at least 3 loci from a panel of 5 gene-associated CpG islands, although this panel has not been standardised leading to discrepancies between studies (Carragher et al., 2010). CpG islands are associated with the start of a gene, and aberrant methylation may lead to gene silencing by promoter repression. It has been suggested that CIMP may be a novel predictive and prognostic marker for colorectal cancer (Shen et al., 2007). Global DNA methylation (hypomethylation) is reduced in many colorectal cancers, and work is ongoing to assess the importance of this finding (Rodriguez et al., 2006).

1.4.7.4 Chromosomal instability (CIN) tumours
Chromosomal instability is found in 85% of colorectal cancers (Grady et al., 2008), with a large meta-analysis demonstrating that CIN is associated with poor prognosis (Walther et al., 2008). Although felt to be important in the progression from
adenoma to carcinoma, the precise mechanisms which cause this instability remain poorly understood.

1.5 Staging of colorectal cancer

The first attempt at uniform staging of rectal cancer was made by the English pathologist Dr. Cuthbert Dukes in 1932 (Dukes, 1932) who classified tumour according to degree of spread. Dukes defined a tumour as stage A if it was confined to the intestinal wall, B if it invaded through the wall or C if there was local lymph node involvement. Metastatic disease was not included in the original grading, as it was considered a terminal diagnosis of limited clinical interest. In a later paper, Duke added stage D to represent distant metastatic spread. However, this was to confirm the dismal prognosis associated with metastatic disease rather than offer improved management (Dukes, 1949). Duke’s demonstration that the stage of the cancer correlated with prognosis led to its adoption for the description of all colorectal tumours.

Improving preoperative radiological imaging and post-operative pathological assessment have led to the development of improved assessment of local invasion and degree of local lymph node spread and the development of more advanced staging systems. The current gold standard staging system is the unified International Union against Cancer/American Joint Committee for Cancer TNM classification.

The TNM classification records the degree of local invasion and spread of a tumour. T records the extent of primary tumour, N records the absence, presence and extent of local lymph node invasion, and M considers evidence of distant metastatic spread (Table 1).
### Table 1 UICC TNM Staging of Colorectal Cancer (Edition 7); (Stewart, 2010)

This staging system assesses degree of local invasion, local and distant metastatic spread of colorectal disease.

<table>
<thead>
<tr>
<th>TNM Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in-situ. Cancer has not breached the muscularis mucosa</td>
</tr>
<tr>
<td>T1</td>
<td>Cancer extending into the submucosa</td>
</tr>
<tr>
<td>T2</td>
<td>Cancer extending into the muscularis propria</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour invading through muscularis propria, into non-peritonealised pericolic/rectal structures</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumour perforating visceral peritoneum</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumour attached to or invading adjacent organs</td>
</tr>
<tr>
<td>Nx</td>
<td>Nodal spread cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1a</td>
<td>Metastasis to 1 regional lymph node</td>
</tr>
<tr>
<td>N1b</td>
<td>Metastasis to 2-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1c</td>
<td>Metastasis to perilymphatic fat, but no true lymphatic spread identifies</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis to 4-6 regional lymph nodes</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis to 7 or more lymph nodes</td>
</tr>
<tr>
<td>Mx</td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No evidence of distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Metastatic spread to 1 distant organ or set of lymph nodes</td>
</tr>
<tr>
<td>M1b</td>
<td>Metastatic spread to more than 1 distant organ or set of lymph nodes</td>
</tr>
</tbody>
</table>

The large number of potential TNM combinations has resulted in the creation of a stage grouping system (Table 2), which groups TNM stages into I-IV with the
proposed benefit that decisions on treatment strategy can be made based on this classification (Carrato, 2008).

<table>
<thead>
<tr>
<th>Stage grouping</th>
<th>TNM Staging</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Tis, N0, M0</td>
</tr>
<tr>
<td>I</td>
<td>T1-2, M0, N0</td>
</tr>
<tr>
<td>IIa</td>
<td>T3, N0, M0</td>
</tr>
<tr>
<td>IIb</td>
<td>T4a, N0, M0</td>
</tr>
<tr>
<td>IIc</td>
<td>T4b, N0, M0</td>
</tr>
<tr>
<td>IIIa</td>
<td>T1-T2, N1, M0 / T1, N2a, M0</td>
</tr>
<tr>
<td>IIIb</td>
<td>T3-T4a, N1, M0 / T2-T3, N2a, M0 / T1-T2, N2b, M0</td>
</tr>
<tr>
<td>IIIc</td>
<td>T4a, N2a, M0 / T3-T4a, N2b, M0 / T4b, N1-N2, M0</td>
</tr>
<tr>
<td>Iva</td>
<td>Any T, Any N, M1a</td>
</tr>
<tr>
<td>IVb</td>
<td>Any T, Any N, M1b</td>
</tr>
</tbody>
</table>

Table 2 UICC/AJCC Stage grouping of TNM staging. The TNM system presents a large number of potential cancer stages. Stage grouping allows grouping of TNM stages, upon which treatment decisions can be made.

1.6 Diagnosis of colorectal liver metastases

Because of its unique location at the head of the portal venous system, the liver is the primary site of metastasis for colorectal cancer. It is thought that haematogenous spread of metastatic disease occurs in a step-wise fashion along the portal tract (Simmonds et al., 2006) and the liver may be the only site of spread in 30-40% of patients with advanced cancer (Hugh et al., 1997). Around 50% of patients with colorectal cancer will develop liver metastases during the course of their disease (Lochan et al., 2007) and around 25% will have liver metastases at the
time of presentation of their bowel pathology (Bengmark et al., 1969). A further 25% of patients who have undergone curative resection of their bowel primary will go on to develop liver metastases at a later date; so called metachronous disease. These patients often have liver metastases detected as part of a routine follow-up program following bowel resection. This is clinically relevant, as it is progression of liver disease rather than progression of primary bowel pathology which determines overall life expectancy (Wood et al., 1976).

1.7 Imaging of colorectal liver metastases

Imaging plays an important role in the detection and management of CRLM. The ability to correctly stage disease in order to guide treatment as well as assess resectability and response to therapy is vital.

Transabdominal ultrasound imaging allows rapid non-ionising assessment of the liver in patients suspected of having CRLM. However, ultrasound scanning is operator-dependent, with a sensitivity of only 66% (Soyer et al., 1992). Transabdominal ultrasound is also limited by fatty infiltration of liver parenchyma; a common finding in post-chemotherapy patients (Pawlik et al., 2007). The development of contrast agents for use with ultrasound has reinvigorated this technique, with studies suggesting 97% of CT detectable lesions are identifiable on contrast enhanced ultrasound (Bernatik et al., 2001).

Intraoperative ultrasound (IOUS) is currently the most sensitive way of detecting lesions, with a sensitivity of between 95-99% in the hands of specialist hepatic surgeons, with the added advantage of being able to detect lesions as small as 2mm (Schmidt et al., 2000; Zacherl et al., 2002). IOUS is now considered the standard of care, and has achieved almost universal usage. Indeed, IOUS has been
shown to change the operative plan in up to 59% of resections where it is employed (Jarnagin et al., 2001).

Modern helical CT techniques have improved pre-operative detection of CRLM with a sensitivity of 60-90% (Ong et al., 2007). Vascular reconstructions of CT scans allow appropriate surgical planning of resections (Sahani et al., 2004), and whole body preoperative scanning allows single session analysis of potential extrahepatic metastatic disease. Multi-detector helical scanning can acquire information about the entire chest and abdomen during a single breath-hold, reducing the risk of breathing artifact (Ong et al., 2007). Rapid introduction of iodine based contrast agents coupled with high-speed scanning allows the entire liver to be visualized in a number of different vascular phases and may improve characterization of hypovascular colorectal liver metastases (Scott et al., 2001).

Indeterminate lesions on CT are further visualized using MRI scanning, which is now considered the most effective imaging modality for detecting and characterizing liver lesions (Kamel et al., 2003). Reported sensitivities for CRLM detected by MRI range between 66-83% (Vidiri et al., 2004) with detection and characterization of liver lesions improved by the addition of liver-specific contrast (Schima et al., 2005).

18-fluorodeoxyglucose positron emission topography (FDG-PET) is a functional imaging technique that exploits the altered glucose metabolism of tumour cells to accumulate a contrast agent (Arulampalam et al., 2004). It is based on the hypothesis that metabolic abnormalities precede anatomical changes in metastatic spread of disease, suggesting PET may allow earlier detection of disease. Recent studies have suggested a sensitivity of between 94-100% (Arulampalam et al., 2004). FDG-PET has the advantage of assessing the entire body, and may detect early recurrent primary disease, as well as low volume peritoneal and lymphatic disease that is not identifiable on CT (Zealley et al., 2001). A study by Huebner et al
suggested that in 29% of cases, patients with CRLM had their management plan altered by further information elucidated through FDG-PET (Huebner et al., 2000). FDG-PET is limited by its inability to accurately provide precise anatomical information. This problem has been addressed by the development of PET-CT, which combines high quality CT images with FDG-PET data, improving tumour detection and lesion localization. Cohade et al reported an improvement in staging and restaging accuracy from 78 to 89% when CT was combined with PET (Cohade et al., 2003). A prospective study by Ruers et al randomized patients to staging by CT-PET or standard CT alone. The number of resections which failed to achieve R0 resection, or patients in whom recurrence was detected within 6 months, was significantly lower in the PET-CT group (28% vs. 45%, P=0.04) (Ruers et al., 2009).

1.8 Surgical management of colorectal liver metastases

Only 10-20% of patients with colorectal liver metastases will be candidates for curative surgery (Adam et al., 2004), but 5 year survival for those who do undergo liver resection is around 50% (Choti et al., 2002; Nordlinger et al., 1994; Yamada et al., 2001; Crowe et al., 2001; Mala et al., 2002; Figueras et al., 2001; Ambiru et al., 1999; Wang et al., 1996; Rees et al., 1997; Wanebo et al., 1996; Schlag et al., 1990; Scheele et al., 1991; Bakalakos et al., 1999) with a recent meta-analysis reporting 10 year survival between 9 - 69% (Taylor et al., 2012). However, 65% who survive for 5 years will experience disease recurrence (Mayo et al., 2009; Jones et al., 2012).

Previously patients with synchronous disease, rectal primary, multiple diffuse metastases, metastases larger than 5cm, disease-free interval of less than 1 year from the diagnosis of primary disease or a high serum CEA were considered to
have a poor prognosis and suitable only for palliative treatment (Poston, 2008). Improved surgical techniques and evolution of neoadjuvant and adjuvant treatments has led to a paradigm shift in selecting patients for surgical resection. Contraindications to resection can now be grouped into two main categories; technical contraindications and oncological contraindications.

1.8.1 Technical resectability

Technical definitions of resectability have evolved. Current consensus in Europe and the US is that disease is resectable if it possible to leave sufficient future liver volume (FLV) to maintain adequate hepatic function, whilst removing all macroscopic disease with negative margins and preserving vascular inflow and outflow (Garden et al., 2006; NCCN 2012).

Deciding what constitutes sufficient liver volume varies depending on the underlying quality of remaining liver parenchyma (known as future liver remnant, or FLR). Twenty-five percent volume (approximately two Couinaud segments) is generally considered adequate. Patients with impaired hepatic function, including those with cirrhosis, steatosis and chemotherapy-associated parenchymal changes, require a larger FLR with significant parenchymal disease considered a barrier to surgery; patients with Childs class B and C liver failure rarely undergo resection. The regenerative nature of liver parenchyma means that significant regrowth takes place after resection and this unique feature means that two stage procedures are feasible with parenchymal regeneration between operations ensuring adequate FLR (Narita et al., 2011). This regenerative capacity is manipulated further using pre-operative portal vein embolisation (PVE) to cause reactive hypertrophy in the proposed FLR (Oussoultzaglou et al., 2006).
Removal of all macroscopic disease traditionally refers to resectability with a negative margin >1mm (R0 resection) (Pawlik et al., 2005). This still remains the gold standard, but there is growing evidence that patients undergoing resection with microscopically positive margins (R1 resection) (de Haas et al., 2008) have better survival than those with macroscopically positive margins (R2 resection). This is thought to be due to hepatic transection devices destroying tissue either side of the transection plane, resulting in obliteration of microscopic residual tumour from the margin.

Anatomical location of metastases may make lesions technically irresectable, as disease cannot be safely removed without sacrificing nearby vessels and drainage structures. The structure itself may be vital (vena cava), or sacrificing the structure may result in the loss of significant parenchyma leaving an insufficient FLV. Development of novel surgical techniques means that a subset of patients with involvement of the vena cava may be candidates for surgery, using total vascular exclusion and ex vivo resection techniques (Garcea et al., 2008). However, expertise in these techniques is limited and so the number of patients who may benefit is small.

1.8.2 Oncological resectability

Although numerous factors are associated with poorer long-term outcome (See section 1.10.2), current oncological contraindications to resection mainly focus on extrahepatic malignancy. However, this is no longer seen as an absolute barrier to surgery with some centres now demonstrating good long-term outcomes in selected groups of patients with resected extrahepatic disease. Survival after partial pneumonectomy for colorectal metastases is similar to that seen after liver resection, with most series quoting a five-year survival in the order of 40-50%, with
low operative morbidity and mortality (Yedibela et al., 2006; Shiono et al., 2005; Pfannschmidt et al., 2003; Saito et al., 2002). Repeat resection of pulmonary metastases may also confer survival benefit, with 5-year survival of 42% reported after repeat resection (Kanzaki et al., 2011). Limited peritoneal and hepatic pedicle nodal disease is also potentially curable, with 5-year survival around 25% following resection (Adam et al., 2011).

Progression on neoadjuvant chemotherapy is a relative contraindication to surgery. A retrospective series of 131 patients who underwent resection for CRLM found that patients whose lesions continued to progress on chemotherapy had a poorer long term prognosis (8% vs. 37% 5-year survival) (Adam et al., 2004). Interestingly, further analysis of data from the same registry suggested patients who underwent liver resection despite progression on chemotherapy in the absence of other high-risk features (synchronous presentation, bilobar disease, multiple (>3) metastases, large (>5cm) metastases, high (>100) preoperative CEA levels) had a 5-year survival of 53.3%. By contrast, resection of progressive disease with high-risk features was associated with a 3-year survival of less than 10%, suggesting progression on chemotherapy alone should not be considered an absolute contraindication to resection (Viganò et al., 2012).

NICE guidance in the UK now recommends that surgery for colorectal liver metastases should be considered if a patient is fit enough, and complete resection can be achieved leaving adequate future liver remnant (Poston et al., 2011). The guidance gave no absolute oncological contraindications to surgery, but suggested that liver resection should not be carried out in the presence of:

- Non-treatable primary tumour
- Widespread pulmonary disease
- Locoregional recurrence
• Uncontrollable peritoneal disease
• Extensive nodal disease, such as retroperitoneal or mediastinal lymph nodes
• Bone or CNS metastases

1.8.3 Techniques of surgical resection

1.8.3.1 Transection techniques

Technological innovations in liver surgery have mainly focused on minimising blood loss during transection of the hepatic parenchyma, as blood transfusion is associated with increased postoperative morbidity and mortality as well as reduced long-term survival (Kooby et al., 2003). Inflow occlusion (Pringle manoeuvre) and low central venous pressure (CVP) anaesthesia minimises blood loss but may cause liver damage by ischaemia and reperfusion injury. Consequently, there has been an interest in devices that facilitate a more bloodless liver transection, obviating the need for inflow occlusion associated with the traditional clamp-crushing technique.

The most popular of these techniques include the ultrasonic aspirating dissector (CUSA) using ultrasonic energy, the Hydrojet using a pressurised jet of water and the dissecting sealer (TissueLink) using radiofrequency energy. These techniques were compared in a randomised controlled trial (Lesurtel et al., 2005) and in a subsequent Cochrane review (Gurusamy et al., 2009). There was little difference demonstrated between the four techniques, though the clamp-crushing technique was found to be associated with faster tissue transection, and lower transfusion requirements. The Cochrane review also found an association with fewer infective complications. Both studies highlighted the significantly reduced cost associated with the clamp-crushing technique, and therefore could not advocate the use of newer techniques in standard practice. A further randomised control trial of
radiofrequency-assisted versus clamp-crushing transection in 50 patients showed a higher rate of postoperative complications in the radiofrequency group (20%), compared to none in the clamp-crushing group (Lupo et al., 2007).

1.8.3.2 Ablation for colorectal liver metastases

Ablative therapy takes numerous forms. Cryotherapy, laser hyperthermia and ethanol injection are decreasing in popularity due to high complication rates or lack of efficacy. Radiofrequency ablation (RFA) and microwave ablation (MWA) offer significant advantages over older ablative techniques and are increasingly used. However, there remains a lack of clarity surrounding the precise role of ablation compared to surgery. Recent American Society of Clinical Oncology (ASCO) guidelines highlighted the wide variation in overall survival and local recurrence rates after ablation, and suggested that in the absence of adequate data resection should remain the gold standard treatment for resectable disease (Wong et al., 2010). Despite these concerns, ablation still has a role as an adjunct to resection. Patients with small volume resectable metastases who are not sufficiently fit to undergo liver resection should be considered for ablation as should those with limited liver metastases who have insufficient liver volume to undergo resection (Oshowo et al., 2003; Jansen et al., 2005).

There is growing interest in the use of ablation alongside systemic chemotherapy for unresectable liver disease. EORTC 40004 compared systemic chemotherapy vs. systemic chemotherapy plus ablation for patients with technically unresectable liver limited disease. 3-year DFS was improved in the combined ablate and chemotherapy arm (27.6% vs. 10%, p=0.03) with a trend towards improved OS (median 45.3 months vs. 40.5, p=0.2) (Ruers et al., 2012). In the French ARF2003 single arm phase II study, 52 patients with unresectable liver limited disease were
treated with a combined ablate and resect strategy using RFA (Evrard et al., 2012). One year local DFS was 46% (95% CI 32-59) whilst 5 year OS was 43% (21-64), demonstrating that ablation and resection can lead to good long-term survival. The evidence now points towards managing stage IV colorectal cancer as a chronic disease condition, where surgery or ablation is performed in the expectation of potentially treatable disease recurrence rather than a single entity which can only be surgically treated once with curative intent or not. Patients who experience recurrence after liver resection can now often be treated with further interventions including surgery or ablation (Jones et al., 2012). Parenchymal sparing techniques (including ablation) preserve functional liver volume, so maximising the opportunity for further liver directed interventions in the future.

1.8.3.3 Laparoscopic liver surgery

Laparoscopic surgery for hepatic neoplasms aims to provide curative resection while minimising complications. There are no randomized controlled trials comparing laparoscopic and open hepatectomy, and so the evidence is based on retrospective series. A meta-analysis of series published between 1998 and 2005 (Simillis et al., 2007) included eight non-randomised studies, reporting on 409 resections of hepatic neoplasms, of which 165 (40.3%) were laparoscopic and 244 (59.7%) were open. Operative blood loss and duration of hospital stay were reduced significantly after laparoscopic surgery. These findings remained consistent when considering studies matched for the presence of malignancy and segment resection. There was no difference in postoperative adverse events and extent of oncological clearance. This paper concluded that laparoscopic liver resection has the potential to reduce operative blood loss and allow earlier recovery with oncological clearance comparable with open surgery.
The largest single-centre experience of laparoscopic resection of CRLM included 83 resections within a series of 133 liver resections (Abu Hilal et al., 2012). The authors reported a median operating time of 210 minutes (30-480 minutes), median blood loss of 300ml (10-3000ml) and a median postoperative stay of 4 days (1-15 days). Severe postoperative bleeding occurred in 5 patients (3.7%) requiring intensive care management or reoperation, and overall serious complications occurred in 16 patients (13%). Microscopically negative margins (R0/R1) were achieved in 96% of patients with CRLM. In 2008 a group of 45 experts in hepatobiliary surgery participated in a consensus conference and concluded that the laparoscopic approach to liver resection is a safe and effective technique for appropriately trained surgeons (Buell et al., 2009).

1.9 Chemotherapeutic management of colorectal liver metastases

In the last 10 years, overall survival (OS) in patients with metastatic CRC has improved substantially (Kopetz et al., 2009) reflecting improved chemotherapeutic manipulation of disease. Before 2000, 5-flourouracil (5-FU) was the only available treatment. With the development of the cytotoxic agents Oxaliplatin and irinotecan, doublet regimens are now considered standard therapy (5-FU/leucovorin/Oxaliplatin as FOLFOX or 5-FU/leucovorin/irinotecan as FOLFIRI).

In the last 5 years, major advances in the management of advanced colorectal cancer have been made by harnessing targeted monoclonal antibodies against extracellular receptors. Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that utilises tyrosine kinase activity for signal transduction with downstream signaling intrinsically involved in multiple biological processes essential for tumour survival (See section 1.4). Cetuximab is a
recombinant human/mouse chimeric antibody that binds specifically to the extracellular domain of the human EGFR, inhibiting this pathway. Panitumumab is a fully human monoclonal antibody with a similar method of action. It is now recognized that patients who have a mutation in the downstream KRAS proto-oncogene are resistant to Cetuximab and Panitumumab therapy, and so KRAS testing is routinely performed prior to commencing treatment (Van Cutsem et al., 2009). Concordance between primary and metastatic tumour is high (>98% for KRAS), allowing analysis of previously resected primary tumour to guide therapy (Vakiani et al., 2012). Improved understanding of the KRAS signaling pathway has identified other common mutations in downstream effectors (including BRAF, NRAS and PIK3CA) which may also confer resistance to anti-EGFR treatments (Roock et al., 2010).

Vascular endothelial growth factor (VEGF) is one of the most important regulators of the dynamic balance between pro and antiangiogenic factors that are crucial for tumour growth and metastasis, with signaling leading to angiogenic proliferation and increased microvascular permeability. Bevacizumab is a humanized monoclonal antibody directed against VEGF receptors. Proposed mechanism of action includes inhibition of vessel development, regression of aberrant tumour vasculature and normalization of tumour perfusion (Ellis, 2006).

1.9.1 Chemotherapy for resectable colorectal liver metastases

Relapse after resection is common, with around two thirds of patients experiencing hepatic recurrence within 2 years (Jones et al., 2012). Chemotherapy aims to maximise the number of long term survivors following such surgery by treating occult disease (Chong et al., 2005; Ellis et al., 2005) and systemic adjuvant chemotherapy has been shown to improve survival after resection of high risk stage
II and all stage III colorectal cancer (Poston et al., 2011). It therefore seems logical that adjuvant therapy after resection of any stage IV disease should also improve long-term outcome. However, the evidence supporting this approach is less than clear.

1.9.1.1 Adjuvant therapy for resectable colorectal liver metastases

In the FFCD AURC 9002 trial (Portier et al., 2006) 171 patients were randomized to liver resection alone or resection followed by adjuvant 5-FU/leucovorin (FU-LV). Due to poor accrual, the trial failed to demonstrate its primary endpoint of improved 5-year overall survival (26.7 vs. 33.5%, ns). The EORTC 0923 trial (Mitry et al., 2008) followed a similar model of resection vs. resection with adjuvant FU-LV and again failed due to poor accrual to demonstrate a significant overall survival (OS) benefit to the combined therapy arm (Median OS 53 vs. 43 months, ns). Both studies were underpowered, and a subsequent pooled analysis demonstrated a median OS of 62.2 months in the chemotherapy arm compared with 47.3 months in the surgery alone arm (HR 1.32, 95% CI 0.95-1.82, p=0.095), with multivariate analysis demonstrating a benefit to adjuvant 5-FU based chemotherapy in terms of both improved disease free survival (DFS) and OS (Mitry et al., 2008). Of note, only 66.7% of patients received all 6 cycles of chemotherapy.

The improved response rates seen after systemic doublet-agent chemotherapy compared to FU-LV suggests that patients might gain a greater benefit from more effective regimens such as FOLFOX/FOLFIRI, but results in stage II and III disease have been mixed. The MOSAIC study demonstrated a clear improvement in long term DFS and OS for stage III patients treated with FU-LV with the addition of Oxaliplatin over FU-LV alone (HR = 0.80; 95% CI, 0.65 to 0.97; P = .023) but this benefit did not translate to patients with stage II disease (André et al., 2009), whilst
the pan-European Trials in Alimentary Cancer (PETACC-3) study failed to demonstrate any improvement in DFS or OS with the addition of irinotecan to adjuvant FU-LV acid in patients with stage III disease (Van Cutsem et al., 2009). For patients with resectable stage IV disease, the evidence is equally limited. A phase III study comparing adjuvant FU-LV versus FOLFIRI following R0 resection of CRLM reported a 2-year disease free survival of 51% and 46% respectively (HR 0.89, 0.66-1.19, p=0.43) (Ychou et al., 2009) similar to that reported in series of resection alone (Morris et al., 2010). The evidence for adjuvant systemic chemotherapy after liver resection may therefore appear marginal. However, variable delivery of any adjuvant regimen may limit any therapeutic advantage, with toxicity, physician or patient preference meaning that many patients do not receive a full treatment course. In the FFCD trial, only 66.7% received complete adjuvant treatment (defined as 85% of the planned dose) whilst the only 53% of patients in the EORTC 0923 trial completed the proposed 6 cycles of adjuvant treatment.

1.9.1.2 Neoadjuvant therapy for resectable colorectal liver metastases

Preoperative treatment offers several potential advantages over adjuvant therapy. It allows the monitoring of chemo-responsiveness in measurable disease, aiding selection of appropriate adjuvant therapy, as well as reducing tumour volume allowing more parenchymal sparing surgery. It also identifies patients with aggressive tumour biology in whom resection may not be appropriate.

A number of early studies suggested that neoadjuvant therapy is safe and well tolerated. A non-randomized phase II study of 20 patients treated with neoadjuvant high-dose 5-FU (2600 mg m$^{-2}$), folinic acid and bi-weekly Oxaliplatin demonstrated successful delivery of 99% of scheduled FU-FA, and 99% of scheduled Oxaliplatin, with a 50% post-operative morbidity and 0% operative mortality (Wein et al., 2003).
Another phase II non-randomized study of neoadjuvant FOLFOX followed by 6 cycles of adjuvant FOLFIRI found no difference in patient compliance between neoadjuvant and adjuvant therapy, with 97% of planned cycles successfully delivered, and no operative mortality (Taïeb et al., 2005). A single-arm study of 6-cycles of neoadjuvant CAPOX (Capecitabine and Oxaliplatin) plus Bevacizumab was able to deliver the planned 6 cycles to 85% of patients, although only 45% received the full schedule. Postoperative morbidity was 21%, with no post-operative deaths, data consistent with matched historical chemonaïve controls (Gruenberger et al., 2008).

Data assessing the efficacy of neoadjuvant therapy on long-term outcome are more limited. A retrospective study of 1471 patients, each with a solitary metachronous resectable colorectal metastasis, compared patients who received at least 3-cycles of FOLFOX/XELOX/CAPOX/FOLFIRI (n=1302) with a group treated with surgery alone (n=169) (Adam et al., 2010). The rate of postoperative complications was significantly higher in the chemotherapy group (37.2% vs. 24%, P = 0.006). At univariate analysis, preoperative chemotherapy did not improve OS (60% at 5 years in both groups); however, postoperative chemotherapy was associated with better OS (65% vs. 55% at 5 years, P < 0.01). Despite the theoretical benefits, the role of perioperative chemotherapy in resectable stage IV colorectal cancer therefore remains unclear.

The multicentre EORTC 40983 study attempted to definitively address this question by randomising 364 patients with resectable liver only CRLM to 12 cycles of perioperative FOLFOX (6 before surgery, 6 after) or surgery alone (Nordlinger et al., 2008). The primary endpoint of the study was 3-year progression free survival (PFS, defined as time from treatment to detection of disease progression), with secondary endpoints including response rate, safety, and OS. In the combined therapy arm only 94% of patients received any preoperative chemotherapy, and only 78.6%
received the full 6 cycles. 152 patients from each arm underwent resection. In the chemotherapy arm, 5% randomised to combined therapy did not undergo surgery; 4 patients were found to have new previously unidentified lesions, whilst 4 (2%) failed resection because of progression of known lesions. Progression on chemotherapy was therefore a rare cause of failed resection. Within the chemotherapy arm, only 115 (63.2%) patients proceeded to post-operative chemotherapy and only 43.9% received the full 6 cycles. For those patients who underwent hepatectomy, the primary endpoint of 3 year PFS was 36.2% in the perioperative chemotherapy arm compared to 28.1% in the surgery alone group (HR 0.77, 0.66-1.00, p=0.041) (Figure 3). This positive result was taken by many as a definitive answer to the benefit of perioperative chemotherapy, and since then it has become the standard of care in many units for patients with resectable CRLM (Nordlinger et al., 2010; Nordlinger et al., 2009). At a median follow up of 8.5 years, nearly half the patients in both arms had died from cancer related causes. However, the addition of perioperative chemotherapy to surgery produced no demonstrable improvement in long term OS (HR 0.87, 0.66-1.14, p=0.303), although there was a non-significant trend towards improved 5-year survival and median overall survival (63.7 months vs. 55 months) in the chemotherapy arm (Nordlinger et al., 2012).
Figure 3 Kaplan-Meier survival curves from EORTC 40983 trial comparing perioperative chemotherapy vs. resection alone for patients with initially resectable CRLM (Nordlinger et al., 2008). The trial showed improved 3-year PFS (primary endpoint). At 8.5-year follow-up, this improvement in PFS did not translate to overall survival benefit (Nordlinger et al., 2012).

1.9.2 Chemotherapy for irresectable disease

A major development in the chemotherapeutic management of advanced disease over the last ten years has been the recognition that there is a subgroup of patients who may not be resectable at presentation but become resectable after chemotherapy.

Resectability rates after chemotherapy for initially irresectable disease vary widely from around 6-60%, the wide variety reflecting patient selection, local approach to resection as well as chemotherapeutic regimen (Poston, 2008). Attempting to
convert irresectable disease into resectable is a worthwhile aim, with 5 year survival around 35-50%, similar to those patients who underwent resection at presentation (Figure 4) (Adam et al., 2001). Radiological response to chemotherapy is known to correlate with resection rate (Folprecht et al., 2005), with studies with defined criteria for resectability reporting higher rates of secondary resection (Figure 5) (Jones et al., 2014). It therefore seems sensible that patients with borderline resectable disease should be treated with the most aggressive regimen possible to provide the best possibility of reaching resection (Nordlinger et al., 2007).

Figure 4 Kaplan-Meier survival curves showing survival following hepatectomy for CRLM comparing those who were initially resectable at presentation to those patients who were considered initially unresectable but were brought to resection using systemic chemotherapy (Adam et al., 2001). This clearly demonstrates long term outcomes superior to systemic chemotherapy for those patients brought to resection.
<table>
<thead>
<tr>
<th>Study</th>
<th>Type</th>
<th>Regimen</th>
<th>No of ptnts.</th>
<th>Overall response rate</th>
<th>p</th>
<th>Patients resected</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPUS</td>
<td>Phase II RCT</td>
<td>FOLFOX+Cetuximab</td>
<td>169</td>
<td>61% (WT)</td>
<td>0.01</td>
<td>9.8%</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOLFOX</td>
<td>168</td>
<td>37% (WT)</td>
<td></td>
<td>4.1%</td>
<td></td>
</tr>
<tr>
<td>CRYSTAL</td>
<td>Phase III RCT</td>
<td>FOLFIRI+Cetuximab</td>
<td>599</td>
<td>59.3% (WT)</td>
<td>0.03</td>
<td>7%</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOLFIRI</td>
<td>599</td>
<td>43.2% (WT)</td>
<td></td>
<td>3.7%</td>
<td></td>
</tr>
<tr>
<td>CELIM</td>
<td>Phase II RCT</td>
<td>FOLFOX+Cetuximab</td>
<td>56</td>
<td>68%</td>
<td>0.23</td>
<td>40%</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FOLFIRI+Cetuximab</td>
<td>55</td>
<td>57%</td>
<td></td>
<td>43%</td>
<td></td>
</tr>
<tr>
<td>POCHER</td>
<td>Single arm phase II</td>
<td>FOLFOXIRI+Cetuximab</td>
<td>43</td>
<td>79%</td>
<td></td>
<td>60.5%</td>
<td></td>
</tr>
<tr>
<td>BEAT</td>
<td>Phase IV prospective study</td>
<td>First line cytotoxic + Bevacizumab</td>
<td>704 liver only</td>
<td>70%</td>
<td></td>
<td>15.2%</td>
<td></td>
</tr>
<tr>
<td>GONO Group</td>
<td>Single arm phase II</td>
<td>FOLFOXIRI+Bevacizumab</td>
<td>57</td>
<td>76%</td>
<td></td>
<td>40% (liver only)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Table showing response rate and resection rate for key trials of cytotoxic agents with the addition of targeted biological agents in patients with initially irresectable metastatic colorectal cancer. WT=KRAS wild type.
Figure 5 Rates of secondary liver resection following systemic chemotherapy. The size of each data point reflects the number of patients included in each study. Blue squares are series that had clearly defined criteria of irresectable disease, and show a highly significant correlation between response rate and secondary resection rate ($R^2=0.62$, $p=0.003$). Red triangles represent series without clearly defined criteria for irresectability, and also show a significant correlation between response and secondary resection ($R^2=0.71$, $p=0.004$). For studies with clearly defined criteria for resectability, rates of secondary resection were much higher for similar rates of radiological response ($p=0.006$). (Jones et al, 2014).

The UK National Institute for Clinical Excellence (NICE) currently recommend the use of 5-FU, leucovorin and Oxaliplatin based regimens (FOLFOX) with or without
Cetuximab as first line therapy for all patients with non-resectable liver-limited disease, with irinotecan based regimens (FOLFIRI) for second line therapy after failure of first line treatment, although efficacy of FOLFIRI is comparable to that of FOLFOX in the setting of metastatic disease (Tournigand et al., 2004; Colucci et al., 2005) with no differences in response rate, PFS or overall survival. The rationale for choosing FOLFOX over FOLFIRI is the perceived increased toxicities associated with systemic irinotecan including early and delayed diarrhoea and severe neutropenia (Innocenti et al., 2004).

Intensive triplet chemotherapy with FOLFOXIRI has been compared with FOLFIRI alone in a phase III randomised controlled trial (Falcone et al., 2007). Response rates were higher after FOLFOXIRI, with a secondary resection rate of 36% in patients with liver-limited disease compared to 12% for those treated with standard FOLFIRI (p=0.017). However, off-target toxicity (mainly severe diarrhoea) was high and so double agent cytotoxic therapy remains the first line

The large phase III CRystal trial randomized 1198 patients to FOLFIRI +/- Cetuximab as first line treatment (Van Cutsem et al., 2009). Retrospective analysis of KRAS status was performed on 1063 patients (Van Cutsem et al., 2011) and found response rates of 57.3% vs. 39.7% for FOLFIRI and Cetuximab compared to FOLFIRI alone in KRAS wild-type patients. By contrast, Cetuximab offered no survival advantage to the KRAS mutant group. Resectability rates for the entire group (irrespective of KRAS status) were 7% in the FOLFIRI + Cetuximab arm, compared to 3.7% in the FOLFIRI arm, with R0 rate of 4.8% vs. 1.7% (p=0.002)

The randomized phase II OPUS trial compared the alternative regimen of FOLFOX +/- Cetuximab in 337 patients with metastatic colorectal cancer, with response rates of 46% vs. 36% respectively (p=0.06) (Bokemeyer et al., 2009). However, subgroup analysis of 315 patients assessed KRAS status demonstrating overall response rate
of 61% in wild type patients compared to 37% in KRAS mutants (p=0.01) (Bokemeyer et al., 2011).

The 2010 phase II CELIM study of neoadjuvant FOLFOX and Cetuximab, or FOLFIRI and Cetuximab, for a more selected group of patients with irresectable metastatic liver only disease found response rates of 85% and 66% respectively. 40% of the FOLFOX and Cetuximab arm underwent resection, compared to 43% of FOLFIRI and Cetuximab. These impressive results suggested that the addition of a targeted biological agent increased the possibility of bringing irresectable liver only disease to resection. In a combined retrospective analysis of both arms, 67 patients with KRAS wild-type tumours achieved a response rate of 79%, (Folprecht et al., 2010). The 2010 phase II POCHER study assessed a chemotherapeutic backbone (irinotecan, Leucovorin, 5-Fluorouracil and Oxaliplatin) alongside Cetuximab in 43 patients with irresectable liver only metastases (Garufi et al., 2010). Despite therapy not being allocated on the basis of KRAS status (the effect of KRAS status on response was unknown at the time of study design), the authors reported a 60% R0/R1 resection rate after a median of 6 cycles, with an objective response rate of 79.1%. Two-year survival was 80.6% in resected patients, compared to 47.1% in those who did not undergo resection (p=0.01). These impressive results highlight the importance of optimal chemotherapeutic manipulation in patients with liver only metastatic disease.

The largest experience of Bevacizumab to date remains the BEAT trial (Van Cutsem et al., 2009). This large phase IV uncontrolled trial assessed the addition of Bevacizumab to first line chemotherapy for patients with irresectable hepatic and extrahepatic metastatic colorectal cancer. 1914 patients were included, with a median PFS of 10.8 months (95% CI 10.4-11.3) and median OS of 22.7 months (95% CI 21.7-23.8). In this unselected group, curative resection was performed in 7.6%. In 704 patients with metastatic disease limited to the liver, resection was
achieved in 15.2%. Two-year survival was 89% in those undergoing resection, compared to 54% in those who did not. This study confirmed a similar efficacy of Bevacizumab in routine clinical practice compared to that observed in a trial setting.

The impressive results reported by the GONO group following systemic FOLFOXIRI led to them performing a single arm phase III trial of FOLFOXIRI with the addition of Bevacizumab (Masi et al., 2010). Treatment was given as first line to 57 patients, with a 74% PFS at 10 months (95% CI 62-85). Curative resection was performed in 40% of those patients recruited with irresectable liver only disease, and this group has now developed a phase III randomised study comparing FOLFOXIRI plus Bevacizumab with FOLFIRI plus Bevacizumab. Preliminary results were presented at ASCO GI 2013, and demonstrated a significantly improved progression free survival (9.5 vs. 11.9 months) and response rate (53% vs. 64%) for the Bevacizumab/FOLFOXIRI arm (Loupakis, 2013).

Interestingly, the addition of Bevacizumab to irinotecan containing regimens appears to improve response rate compared to Oxaliplatin based regimens, and so when conversion to resectability is the primary aim of therapy this combination may become first-line therapy in KRAS mutant patients (Fuchs et al., 2007; Hurwitz et al., 2004). A summary of these response and secondary resection rates is outlined in table 3.

1.9.3 Chemotherapy-induced liver injury

Growing evidence suggests that preoperative chemotherapy is associated with agent-specific patterns of chemotherapy associated liver injury (CALI, Figure 6) including steatosis (5-FU/irinotecan), (Parikh et al., 2003) steatohepatitis (5-FU/irinotecan) (Fernandez et al., 2005) and sinusoidal obstructive syndrome
(Oxaliplatin) (Rubbia-Brandt et al., 2004). In addition, there has been concern about the perioperative impact of this hepatotoxicity.

![Image](image-url)

Figure 6 Representative photomicrographs of chemotherapy associated liver injury (A) Sinusoidal obstructive syndrome (SOS) produced by administration of systemic Oxaliplatin, showing dilated sinusoids suggestive of vascular congestion. (B) Steatohepatitis and steatosis induced by systemic irinotecan, with characteristic micro and macrosteatosis, inflammatory cell congregation and hyaline bodies (black arrows). (Both x30 magnification) (Figure adapted from Poston 2014)

EORTC 40983 reported a moderate increase in reversible morbidity in the chemotherapy arm (25% vs. 16%, p=0.04), but 30-day mortality for both groups remained 1%. Conflicting results have been published since then (Parc et al., 2000; Ryan et al., 2010) with Vauthey et al (Vauthey et al., 2006) reporting a clear association between the administration of systemic irinotecan and steatohepatitis (20.2% vs. 4.4% in chemonaïve patients, p<0.01, OR 5.4 [95% CI 2.2-13.5]), which translated into a significant increase in 90-day post-operative mortality (14.7 vs. 1.6%, p=0.001, OR 10.5 [95% CI 2.0-36.4]). There is a clear correlation between number of cycles of chemotherapy, degree of liver damage and perioperative risk.
(Karoui et al., 2006). However, this risk appears to be mitigated by allowing an appropriate interval (usually 6 weeks) between the final cycle of systemic therapy and surgery (Welsh et al., 2007). Interestingly, a high quality systematic review and meta-analysis suggested that the use of Bevacizumab alongside FOLFOX may offer a protective benefit, significantly reduced the risk of sinusoidal injury (relative risk 0.34; 95 % CI 0.15-0.75) (Robinson et al., 2012). However, the precise mechanism behind this remains unclear.

As well as concerns about the perioperative impact, there is growing interest in the long-term oncological implications of chemotherapy associated liver injury with Tamandl et al (Tamandl et al., 2011) reporting shorter DFS (HR 2.05; 95% CI 1.23-3.39, P = .005) and OS (HR 2.90; 95% CI 1.61-6.19, P < .001) in patients with sinusoidal obstructive syndrome caused by preoperative Oxaliplatin.

1.9.4 Radiological assessment of chemotherapeutic response

Imaging plays a key role in the assessment of response to treatment. A major international collaboration led to the definition of the Response Evaluation Criteria in Solid Tumours (RECIST) criteria to standardize reporting of response rates to therapy (Therasse et al., 2000). All radiologically measurable lesions (longest diameter >10mm on spiral CT) up to a maximum of 5 per organ and 10 in total are identified and recorded as target lesions. A sum of baseline longest dimensions (LD) is then calculated (baseline LD). Response of target lesions is assessed as complete response (CR, disappearance of all lesions), partial response (PR, at least a 30% reduction in the sum of LD of target lesions compared to baseline LD), progressive disease (PD, at least 20% increase in the sum of LD compared to best LD since treatment began) and stable disease (SD, neither sufficient shrinkage or progression to classify as PR or PD).
In 2009, these criteria evolved into modified RECIST version 1.1 which reduced the maximum number of target lesions from ten to five, with a maximum of two per organ. Progressive disease (PD) of target lesions now required not only a $> 20\%$ increase in the sum of the longest diameter (SLD) from the nadir but also a $> 5 \text{ mm}$ absolute increase in the SLD. PD of non-target lesions could now only be applied if the increase in non-target lesions represented a change in overall tumour burden.

Attempts to correlate pathological response with radiological response have proved difficult (Grothey et al., 2008) with over 80\% of lesions demonstrating complete radiological response containing viable tumour on histological examination (Benoist et al., 2006). It has now been recognized that that change in tumour size may not accurately reflect the efficacy of therapy, shifting the focus toward new methods of evaluation including the assessment of tumor morphology, vascularity and cellular integrity defined by the degree of extracellular contrast enhancement. The European Association for the Study of the Liver (EASL) officially recommended the use of lesion enhancement on contrast-enhanced CT as the standard modality to determine treatment response of HCC after locoregional therapy (Bruix et al., 2001) and new guidelines are being developed to describe the different response of metastatic colorectal lesions to modern biologic therapies such as Bevacizumab (Chun et al., 2009).

The role of PET imaging in the assessment of chemotherapeutic response and immediate post-chemotherapy period remains unclear. Glazer et al. (Glazer et al., 2010) found that PET scan within 4 weeks of finishing neoadjuvant chemotherapy is associated with a negative predictive value of 13.3\% and a positive predictive value of 94.3\%. Sensitivity was 89.9\% and specificity was 22.2\%, with an accuracy of 85.5\%. A further study assessed correlation between complete response to neoadjuvant therapy on PET-CT and pathological response after resection.
Complete pathologic response was found in only 15% of treated lesions. Of seven lesions showing complete metabolic response of PET-CT, six still contained viable tumor. It therefore seems that complete metabolic response on FDG-PET after neoadjuvant chemotherapy is an unreliable indicator of complete pathologic response (Tan et al., 2007).

1.9.5 Pathological assessment of chemotherapeutic response

It is well recognised that patients who exhibit a good pathological response to chemotherapy have better overall survival (Rubbia-Brandt et al., 2007). A study by Blazer et al found complete pathological response to chemotherapy was associated with a 5-year survival of 75% (Figure 7) (Blazer et al., 2008). Complete response occurred in a small minority of patients (9%) but survival was significantly better than those with less response (75% 5-year survival, compared to 56% for those with a major response and 33% for those with a minor response, p=0.037).

This finding was supported by Adam et al, who found patients exhibiting complete pathological response had a 5 year survival of 76%, compared to 45% for those without (Adam et al., 2008). Interestingly, there is now growing evidence that chemotherapy-associated change seems to be represented by fibrotic involution rather than necrosis (Poultsides et al., 2012).

Complete pathological response is an impressive example of the effectiveness of modern chemotherapeutic regimes. Although oncologically desirable, it creates surgical difficulties. The correlation between complete radiological and pathological response is not clear, with many lesions showing complete radiological response containing viable tumour. Trying to locate a lesion that has disappeared is difficult, and results in patients undergoing blind resection on the basis of the last known location of that lesion. The difficulties associated with disappearing lesions
highlights the importance of combined surgical and oncological planning to optimise the chemotherapeutic manipulation of disease, as well as the timing of any intervention. Improved pre-operative assessment of pathological response by imaging will become increasingly important in deciding which patients can be managed with a “watch and wait” policy.

Figure 7 Kaplan-Meier survival curves of patients treated with systemic neoadjuvant chemotherapy stratified by pathological response grade. Pathological response is stratified as minor, moderate or complete depending on degree of residual tumour (Adapted from Blazer et al., 2008).
1.10 Prognostic and predictive markers for colorectal liver metastases

1.10.1 Clinicopathological staging as a predictor of outcome

Figure 8 Kaplan–Meier survival curves from a population of 110,000 UK patients diagnosed with primary colorectal cancer between 1998 and 2003 with 5-year follow up stratified by disease stage. Patients with non-resected stage IV disease had a dismal prognosis, compared to those with resected stage IV disease who had long-term survival comparable to those who present with stage III disease. (Adapted from Morris et al., 2010).

The wide patterns of colorectal disease, coupled with varied tumour biology and evolving management, help explain the wide variations in survival between disease subgroups. However, these varying outcomes are not currently reflected in the existing staging system for metastatic colorectal cancer. A recent UK study reviewing 110,000 patients with CRC found a 5-year survival for stage III disease of approximately 45% whilst patients with stage IV disease who underwent curative liver resection had a 5-year survival of over 50% (Figure 8) (Morris et al., 2010). That is, patients with curatively resected stage IV disease had better overall survival.
than patients with stage III disease. The current staging system has no way of reflecting this.

1.10.2 Prognostic scoring systems as a predictor of outcome

It is clear that the boundaries of what is technically resectable continue to evolve, and the number of patients who initially present with irresectable disease and are converted into resectable disease by chemotherapy continues to grow. What remains unclear is how to identify patients in whom resection, whilst technically and oncologically reasonable, is not appropriate. Improved understanding of tumour biology is leading to increased interest in novel biomarkers to predict disease progression and survival (Ohtani, 2007; Tejpar et al., 2010). In the future, detailed analysis of individual tumour behaviour may offer a better predictor of outcome and response to therapy than traditional clinicopathological factors. High-throughput genomic and proteomic technologies suggest this would be technically and economically viable. However, large-scale validation of potential markers is required before they can be adopted for clinical practice.

Currently, a number of scoring systems are used that attempt to classify patients by clinical prognosis based on existing preoperatively identifiable factors. The most popular of these were produced by Fong (Fong et al., 1999), Nordlinger (Nordlinger et al., 1996) and Rees. (Rees et al., 2008) The Fong classification (Clinical Risk Score) demonstrated a poor prognosis associated with synchronous presentation, rectal primary, multiple diffuse metastases, metastases larger than 5cm, disease-free interval of less than 1 year from the diagnosis of primary disease or a high serum CEA, and this score is the most widely used owing to its ease of use. Nordlinger’s classification ranges from 0-7 with 1 point being awarded for each of the following adverse risk factors.
• Extension into serosa of primary tumour
• Lymphatic spread of the primary tumour
• Delay from primary tumour to resection <24 months
• Number of liver metastasis in preoperative imaging
• Largest size of liver metastasis in preoperative imaging ≥ 5.0 cm
• Preoperatively estimated clearance of normal parenchyma resected with liver metastasis <1cm.
• Age ≥60

These two scoring systems have been compared (Table 4) (Zakaria et al., 2007; Merkel et al., 2009), with the Clinical Risk Score proving to be more appropriate for use in clinical practice and better at differentiating between groups. Both scoring systems exclude patients with extrahepatic disease, and fail to take into account many other known adverse risk factors meaning that their clinical utility may become increasingly limited (Taylor et al., 2012).
<table>
<thead>
<tr>
<th>Nordlinger Prognostic Index</th>
<th>Fong Clinical Risk Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extension into serosa of primary tumour</td>
<td>Nodal status of primary</td>
</tr>
<tr>
<td>Lymphatic spread of the primary tumor</td>
<td>Disease free interval from primary to metastases &lt;12 months</td>
</tr>
<tr>
<td>Delay from primary tumour to resection &lt;24 months</td>
<td>Number of tumours &gt;1</td>
</tr>
<tr>
<td>Number of liver metastasis in preoperative imaging</td>
<td>CEA &gt;200 ng/ml</td>
</tr>
<tr>
<td>Largest size of liver metastasis in preoperative imaging ≥ 5.0 cm</td>
<td>Size of largest tumour &gt;5cm</td>
</tr>
<tr>
<td>Preoperatively estimated clearance of normal parenchyma resected with liver metastasis &lt;1cm.</td>
<td></td>
</tr>
<tr>
<td>Age ≥60</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Comparison of Nordlinger Prognostic Index and Fong Clinical Risk Score.

Rees (Rees et al., 2008) proposed a scoring system that could be used in either the preoperative or postoperative setting. In this risk prediction model, points were allocated up to a maximum of 30 based on primary lymph node status, primary tumour differentiation, CEA, number and size of lesions and resection margin (Table 5). Patients with a score of 0, 10, 20 and 30 on preoperative scoring had 5-year survival rates of 66%, 35%, 12% and 2% respectively (Figure 9). This compared very well with the scores determined postoperatively. However, the complexity of this scoring system has limited its uptake as a clinical tool.
Figure 9 Kaplan-Meier plot showing survival after primary liver resection for CRLM stratified by Basingstoke Prognostic Index (Rees et al., 2008).

Despite the differences between these 3 prognostic indices, there is significant agreement and overlap between the key independent predictors of poor long-term outcome.
### Basingstoke Predictive Index

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Preoperative</th>
<th>Postoperative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Tumour Lymph node Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Primary Tumour differentiation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Poor</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td><strong>CEA at hepatectomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6 ng/mL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6-60 ng/mL</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;60 ng/mL</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Number of hepatic metastasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>&gt;3</td>
<td>4</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Largest Tumour diameter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5cm</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5-10cm</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>&gt;10cm</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td><strong>Hepatic resection Margin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>n/a</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>n/a</td>
<td>11</td>
</tr>
<tr>
<td><strong>Extrahepatic disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5 The Basingstoke Prognostic Index. This scoring system can be used in either the preoperative or postoperative setting. Points are allocated (up to 30) based on clinicopathological factors, with a higher score associated with worse long-term outcome (Figure 9).
1.10.3 Biomarkers for colorectal cancer

Despite improved treatments for colorectal cancer, many patients receive toxic chemotherapies from which they derive no benefit. This has led to a drive to develop novel biomarkers for cancer that allow both the clarification of disease prognosis and prediction of patient response to therapy. A biomarker is a measured characteristic which can be used as an indicator of a biological state or condition. Putative oncological biomarkers include mRNA, DNA, protein, circulating tumor cells (CTC) or tumor-derived nucleic acids that can be used to stratify patients for treatment benefit within clinical trials, prognosticate patient outcome, or predict and/or monitor response to therapy (Sikorski et al., 2010). These biomarkers may be measured in easily obtainable body fluids such as blood, bile or urine, or through more invasive techniques requiring tumour tissue. A prognostic biomarker provides insight into a patients overall cancer outcome, regardless of any therapy. Detection of a prognostic marker may guide decision making when it comes to recommending a certain treatment e.g. adjuvant treatment after surgery, but does not predict treatment response. A biomarker with predictive value gives information on the effectiveness of a therapeutic intervention in a patient.

1.10.3.1 Existing predictive and prognostic biomarkers

Multiple meta-analyses have assessed the value of genetic and epigenetic factors as prognostic markers of outcome, as well as predictive markers to help guide drug selection. This is of particular value in selecting expensive and potentially toxic treatments for heterogeneous disease. A summary of current prognostic and predictive molecular biomarkers is outlined in table 6 and 7. Although offering great potential, very few markers are validated and used in clinical practice. However, this seems likely to change as targeted agents and clinical trials with biomarker validation as a key endpoint become more common.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mutation Frequency</th>
<th>Prognosis</th>
<th>Evidence</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsatellite instability (MSI)</td>
<td>15%</td>
<td>Favourable</td>
<td>Strong</td>
<td>Testing available but not widely used</td>
</tr>
<tr>
<td>Chromosomal instability (CIN)</td>
<td>70%</td>
<td>Unfavourable</td>
<td>Strong</td>
<td>No readily available test</td>
</tr>
<tr>
<td>18qLOH/SMAD4 loss</td>
<td>50%</td>
<td>Unfavourable</td>
<td>Moderate</td>
<td>No readily available test</td>
</tr>
<tr>
<td>BRAF V600E</td>
<td>10%</td>
<td>Unfavourable</td>
<td>Moderate</td>
<td>Test available, but insufficient evidence to justify clinical use</td>
</tr>
<tr>
<td>KRAS Codon 12/13</td>
<td>40%</td>
<td>Unfavourable in advanced disease</td>
<td>Limited</td>
<td>Testing available, but prognostic value unclear</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>20%</td>
<td>Possibly unfavourable</td>
<td>Limited</td>
<td>Testing not readily available</td>
</tr>
</tbody>
</table>

Table 6 Prognostic biomarkers in colorectal cancer (Adapted from Pritchard et al., 2011).
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mutation Frequency</th>
<th>Drug selection</th>
<th>Evidence</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS codon 12/13</td>
<td>40%</td>
<td>Predicts resistance to anti-EGFR therapy</td>
<td>Strong</td>
<td>Validated, in clinical use</td>
</tr>
<tr>
<td>BRAF V600E</td>
<td>10%</td>
<td>Probably predicts resistance to anti-EGFR</td>
<td>Moderate</td>
<td>In clinical use, not fully validated</td>
</tr>
<tr>
<td>PIK3CA mutations</td>
<td>20%</td>
<td>May predict resistance to anti-EGFR</td>
<td>Limited</td>
<td>No readily available test, not in clinical use</td>
</tr>
<tr>
<td>PTEN loss</td>
<td>30%</td>
<td>May predict resistance to anti-EGFR</td>
<td>Limited</td>
<td>No readily available test, not in clinical use</td>
</tr>
<tr>
<td>Microsatellite instability (MSI)</td>
<td>15%</td>
<td>May predict adverse outcome with 5-FU and improved outcome with irinotecan</td>
<td>Moderate</td>
<td>No routine clinical use</td>
</tr>
<tr>
<td>18qLOH/SMAD4 loss</td>
<td>50%</td>
<td>May predict resistance to 5-FU</td>
<td>Moderate</td>
<td>Not in routine clinical use</td>
</tr>
<tr>
<td>Topo1 Low</td>
<td>50%</td>
<td>May predict resistance to irinotecan</td>
<td>Limited</td>
<td>Not in routine clinical use</td>
</tr>
</tbody>
</table>

Table 7 Predictive biomarkers in colorectal cancer (Adapted from Pritchard et al., 2011).
1.11 Locoregional management of colorectal liver metastases

1.11.1 Hepatic arterial infusion

The unique blood supply of the liver, with portal flow supplying healthy hepatic parenchyma and arterial flow supplying metastatic disease, has led to the concept of delivering liver-only chemotherapy in an effort to increase metastatic exposure to the agent whilst reducing systemic dose and off-target side-effects. Most cytotoxic drugs have a steep dose response curve and therefore a higher cytotoxic effect is obtained by high local concentrations (Widder et al., 1979). Initial interest focused on hepatic arterial infusion (HAI) as a replacement for systemic adjuvant chemotherapy. A catheter is inserted at laparotomy into the umbilical vein remnant, through which a portable pump delivers an infusion of chemotherapeutic agent. Kerr et al randomized 290 patients with liver limited irresectable metastatic colorectal cancer to receive fluorouracil and folinic acid either as continuous intravenous infusion or delivered by HAI. 37% of patients allocated to HAI did not commence treatment because of catheter related complications, with a further 29% stopping before completing 6 cycles of therapy because of subsequent catheter failure. For those completing 6 cycles, overall survival was comparable between intravenous and HAI infusion (14.7 vs. 14.8 months, p=0.79) (Kerr et al., 2003). These findings were supported by a high quality meta-analysis by Mocellin et al (Mocellin et al., 2007) which found no benefit of HAI compared to systemic chemotherapy. However, interest continues in its use as an adjunct to first line systemic therapy.

A phase III randomised trial considering FUDR (an analogue of 5-FU) HAI as adjuvant treatment alongside systemic 5-FU after resection of CRLM showed a significant improvement in 2-year recurrence free survival of 90%, compared to 60% for those receiving systemic chemotherapy alone (Kemeny et al., 1999). At a median follow up of 10.3 years, 10 year survival rates were 41.1 percent and 27.2
percent respectively (Kemeny et al., 2005). A further phase I study assessed pharmacokinetics of irinotecan in heavily pretreated patients with liver metastases and demonstrated higher activation of the prodrug into its active metabolite after intra-arterial infusion compared to intravenous infusion (van Riel et al., 2002). However, this did not translate into a clinical benefit in follow-on phase II studies.

These impressive results stimulated interest in whether the high response rates to HAI could be harnessed to bring more patients to resection, with a systematic review of neoadjuvant hepatic arterial infusion alongside systemic chemotherapy in irresectable CRLM reporting secondary resection rates between 6-47% in unselected series (Pwint et al., 2010). Kemeny et al (Kemeny et al., 2009) published data of an early phase Ib trial of 49 patients treated with systemic FOLFOX with FUDR HAI in irresectable liver-only disease. Again, HAI was used as an adjunct to systemic chemotherapy. They reported an 8% complete radiological response rate, an 84% partial response and a 47% conversion to resectability, which increased to 57% in chemonaïve patients (Shitara et al., 2006; Gallagher et al., 2007).

Although these results are promising, technical and toxic complications (Kulaylat et al., 2010) including chemical hepatitis, biliary sclerosis, arterial and venous thrombosis and catheter displacement have restricted its role to preliminary research work. The Memorial Sloan Kettering group reported their experience of 544 consecutive insertions of HAI pump, and found a 16% failure rate within 2 years of insertion (Allen et al., 2005).

1.11.2 Trans-arterial chemoembolisation (TACE)

Attempts to minimise the technical aspects of liver-only treatment has led to the development of novel drug delivery methods. Although primarily developed for use
in irresectable hepatocellular carcinoma (Llovet et al., 2003) interest is growing in TACE as a tool for the management of CRLM.

Conventional TACE (cTACE) involves the injection of chemotherapeutic agent into blood vessels supplying tumour, followed by embolic material in an effort to occlude the feeding blood vessels. This approach offers advantages. The ischaemic effect of the embolisation will lead to tumour infarct resulting in cell death, although this alone is unlikely to destroy all the tumour as tumour phenotypes tend towards hypoxia-resistant systems (Huerta et al., 2009). Hypoxic damage will however increase vascular permeability, encouraging diffusion of chemotherapeutic agents from the vascular compartment into tumour (Wallace et al., 1990). Reduced perfusion after embolisation reduces chemotherapeutic washout, increasing local concentrations of the drug and increasing total tumour exposure.

Kato et al (Kato et al., 1981) first described the use of cTACE in the management of metastatic colorectal disease in 1981. Since then, a variety of embolic devices loaded with different drugs have been trialed (Fujimoto et al., 1985; Sasaki et al., 1990; Meakem et al., 1992; Maeda et al., 1993; Voigt et al., 2002; Salman et al., 2002). The heterogeneous nature of these studies as well as the extensive time-period makes direct comparison difficult, but a critical review by Vogl et al reported morphological response (complete/partial response on RECIST) of CRLM to cTACE of 14 – 76 % (Vogl et al., 2007).

Vogl et al also performed a large prospective study investigating the role of repeat cTACE for the palliative treatment of patients with CRLM (Vogl et al., 2009). 463 patients with liver limited metastatic colorectal cancer failing second-line systemic chemotherapy were treated with 4-weekly cTACE. Embolisation was performed using lipidiol or starch microspheres, with a combination of mytomycin C, gemcitabine and irinotecan depending on previous chemotherapeutic regimen and
response. 14.7% of patients exhibited partial response, 48.2% had stable disease and 37.1% had progressive disease. 1- and 2-year survival after chemoembolisation was 62% and 28% respectively, with median survival from diagnosis of 38 months, compared to 7-8 months for palliative treatment. They concluded that cTACE offered an effective and minimally invasive therapy for the palliative management of CRLM.

Tsuchiya et al (Tsuchiya et al., 2007) reported 27 cases of irresectable CRLM treated with cTACE using irinotecan and degradable starch microspheres. cTACE was performed 47 times, with 9 patients (33%) being downstaged to surgery. Response rate was 59%, with a 3-year survival of 20%.

Ceelen et al (Ceelen et al., 1996) assessed 23 patients in a treat and resect experimental study, 14 of whom were treated preoperatively with cisplatin/iodized oil/gelfoam chemoembolisation. cTACE had no effect on operating time, transfusion requirements or post-operative complications. Although this was a small study, a significantly lower rate of recurrence was seen in those patients treated preoperatively with embolisation (8% vs. 67%), suggesting preoperative cTACE might improve survival post-resection.

1.11.3 Drug eluting beads for TACE (DEB-TACE)

Drug eluting beads (DEB, marketed as DC Bead in Europe) are compressible beads produced from polyvinyl alcohol (PVA) hydrogel that can be loaded with irinotecan, and offer a theoretical advantage over hepatic arterial infusion and conventional TACE because of simplified delivery (embolisation and chemotherapy are combined, with no need for a pump) and offers the potential to add locoregional irinotecan to systemic FOLFOX with the aim of achieving comparable response rates to that seen after FOLFOXIRI whilst minimising morbidity. DC Bead loaded
with irinotecan is CE Marked in Europe for the treatment of metastatic colorectal cancer. DC Bead does not yet have market approval in the US to be used in combination with irinotecan but is used in FDA approved investigational clinical trials, and has previously shown impressive results in both chemo-naïve and heavily pre-treated irresectable patients (Martin et al., 2010; Martin et al., 2012).

DC beads are compressible embolic microspheres produced from polyvinyl alcohol (PVA) hydrogel and are available in 3 sizes (100-300 µm, 300-500 µm and 500-700 µm). Bead size is selected by the administering radiologist based on the size of the targeted vessels. The variation in diameter within each size allows the bead to be carried to the point where the size of the microsphere determines the point of blockage, as opposed to the more proximal embolisation caused by particle aggregation when using other embolic materials.

The beads are supplied in a glass vial. Loading is performed immediately prior to delivery by adding an irinotecan solution to the vial. The beads contain multiple negatively charged sulfonate groups which form ionic bonds with positively charged drugs, allowing for rapid loading (Lewis, 2009). Liquid chromatographic analysis has shown that loaded beads are stable for up to seven days after loading. All of the drug can be recovered from the bead i.e. no irreversible chemical reaction occurs between the drug and the bead (Lewis et al., 2006). Bead loading with irinotecan is affected by the existence of two forms of the drug; the active lactone and inactive carboxylate form, with the ratio between the two dependent on local pH. The lactone form carries more of an overall positive charge, and so is preferentially bound to the bead. As the lactone form binds to the bead, more carboxylate form is converted to lactone to maintain the equilibrium. When the drug disassociates from
the bead within tissue, it is the active lactone form which is released (Tang et al., 2008).

Drug-bead interaction modeling has shown that the loading time is both bead-size and drug-dose dependent, ranging between 1 and 12 minutes, with a maximum irinotecan loading of 50-60 mg ml\(^{-1}\) for beads for all sizes (Tang et al., 2006). Loading beads with drug has no effect on handling or compressibility, making them appropriate for selective embolisation using narrow gauge radiological catheters (Lewis et al., 2006).

1.11.3.1 Animal and preclinical assessment of DEBIRI-TACE in metastatic liver disease

Tang et al performed preliminary animal modeling using porcine hepatic arterial embolisation in four groups of animals using 100-300 \(\mu m\) control beads, 100-300 \(\mu m\) irinotecan loaded beads, 700-900 \(\mu m\) irinotecan loaded beads and intra-arterial injection of irinotecan alone. Irinotecan loaded beads were loaded at a concentration of 50 mg ml\(^{-1}\). Plasma samples were taken over 90 days, and histopathology was performed after animal sacrifice at 30 and 90 days. Maximum plasma levels of irinotecan were 70-75\% lower for irinotecan bead embolisation compared with arterial administration, with both bland and irinotecan embolisation showing necrotizing vasculopathy in keeping with ischaemia. Interestingly, C\(_{\text{max}}\) was 1791+/-521 ng ml\(^{-1}\) for the 100-300 \(\mu m\) beads compared to 1479+/-703 ng ml\(^{-1}\) for the 700-900 \(\mu m\) beads, suggesting bead size does not affect drug release. (Tang et al., 2006). Preclinical animal modeling using a WAG/Rij rat model with implanted metastases from a CC531 colorectal cancer cell line directly compared bland embolisation, irinotecan loaded bead embolisation and doxorubicin embolisation. Dose delivery was calculated by body weight, and so embolisation was not performed to stasis. Bland embolisation demonstrated no reduction in tumour
burden, compared to irinotecan loaded DC beads which demonstrated a 42% reduction in tumour burden (Eyol et al., 2008).

Preliminary human safety and pharmacokinetic data on irinotecan bead therapy was produced by Professor Thomas Vogl at the Universität Frankfurt am Main (See appendix 2). Ten heavily pretreated patients with irresectable CRLM were treated with intra-arterial administration of irinotecan loaded beads at a median dose of 85 mg (range 36-143 mg). The mean peak plasma dose of irinotecan was 194 ng ml\(^{-1}\) (SD 124), whilst the mean peak level of SN-38 was significantly lower at 17.2 ng ml\(^{-1}\) (SD 11.3). The mean \(T_{\text{max}}\) of irinotecan was 2 hours, whilst the mean \(T_{\text{max}}\) for SN-38 was 1 hours (Figure 10).

![Figure 10 Preclinical analysis of serum drug and metabolite concentrations after treatment with DEBIRI. Mean (± standard deviation) plasma concentrations of irinotecan and its metabolite SN-38 in patients treated with irinotecan-loaded DC bead microspheres at a nominal dose level of 400 mg irinotecan from preliminary pharmacokinetic study by Prof Thomas Vogl (See appendix 2). Concentrations of irinotecan and SN-38 are significantly lower than those seen after IV infusion at comparable doses (See section 1.12.4).]
However, accuracy is limited by the lack of sampling between 1 and 2 hours. Inter-individual variability in peak drug concentrations was greater than 50% for irinotecan (co-efficient of variation 63.8%) and SN-38 (co-efficient of variation 67.8%).

1.11.3.2 Clinical assessment of DEBIRI-TACE in irresectable CRLM

A pilot study assessed the safety and feasibility of DEBIRI-TACE in patients with heavily pretreated unresectable CRLM (Eichler et al., 2012). Eleven patients with progressive disease after second-line systemic Oxaliplatin or irinotecan based chemotherapy received up to 4 cycles of DEBIRI-TACE at 3-weekly intervals (median total dose 293mg). Only minor adverse events were noted, with 63% of patients developing abdominal pain, nausea and vomiting. No irinotecan-related toxicities were observed. DEBIRI-TACE was technically feasible in all cases, with a 64% disease response rate after 9 weeks. Median time to progression was 154 days from first treatment.

An Italian group published preliminary results from a small phase II safety and efficacy study on ten patients with irresectable CRLM treated with irinotecan loaded beads (Aliberti et al., 2006) with a demonstrable radiological response in 70% of treated lesions. All patients experienced right upper quadrant and shoulder tip pain requiring analgesia. Final results of this study published in 2007 (Fiorentini et al., 2007) included 20 patients treated with DEBIRI-TACE. All patients had failed previous treatment with FOLFOX+/-FOLFIRI. Six patients received one DEBIRI-TACE, 8 patients received two DEBIRI-TACE and 6 received three DEBIRI-TACE at 3-weekly intervals each at a nominal dose of 100mg. Fifteen patients were alive at the time of study reporting, with a median follow-up of 200 days. All patients experienced pyrexia, right upper quadrant pain and nausea after embolisation. None of the patients demonstrated systemic toxicity to irinotecan.
The same group progressed to a larger single arm phase II study assessing tumour response and survival in heavily pre-treated patients with CRLM (Aliberti et al., 2011). Eighty-two patients who had failed first line systemic therapy were treated with 185 DEBIRI-TACE (median 2.2 per patient) at 3 weekly intervals. Post-embolisation syndrome was frequently observed, characterized by right upper quadrant pain, fever, nausea and transient increase in transaminases and reported after the majority of embolisations. There was a 78% 3-month lesional response rate, with a median duration of response of 6 (range 3-10) months. Median survival was 25 (range 6-34) months with progression free survival at 8 (range 4-16) months, comparable with first line systemic therapies.

These impressive results led the same group to develop a phase III randomized control study comparing DEBIRI-TACE versus systemic FOLFIRI as first-line treatment for liver limited metastatic colorectal cancer (Fiorentini et al., 2012). However, patients were eligible for inclusion if they had received systemic therapy more than 3 months prior to enrollment in the study and in fact many of these patients had been heavily pretreated. Patients were randomized to FOLFIRI or DEBIRI based on percentage liver involvement, lines of previous therapy, weight loss, KRAS status and p53 expression. The systemic therapy arm received FOLFIRI for 4 months of treatment whilst the DEBIRI arm received drug eluting beads loaded with irinotecan twice a month for the same time period. Overall response rate after DEBIRI-TACE was 69%, compared to 20% after FOLFIRI with two-year survival of 56% for the DEBIRI-TACE group, compared to 32% for the FOLFIRI group (p=0.03). All patients had extrahepatic disease progression at 2-year follow-up highlighting the poor biology of this cohort of patients. Surprisingly, a significant delay (4 months) in time to extrahepatic progression was noted in the DEBIRI-TACE arm that appears counterintuitive following liver-only therapy. Subgroup analysis by KRAS status found that wild-type patients had a median survival of 26 months after
DEBIRI-TACE, compared to 19 months for KRAS mutants. Comparison of toxicity between the two arms demonstrated grade 3 neutropenia in 4% and 44% (p<0.0001), diarrhoea in 6% and 18% (p=0.07) and mucositis in 1% and 20% (p=0.00002) of the DEBIRI and FOLFIRI groups respectively. This trial suggested that DEBIRI-TACE offers improved response rate, overall survival and reduced toxicity when compared to systemic FOLFIRI.

A multi-institute registry of patients with irresectable CRLM treated with irinotecan DEBIRI-TACE considered 55 patients who underwent 99 embolisations for irresectable colorectal liver metastases (Martin et al., 2010). The treatment approach in this registry involved whole lobe embolisation, rather than selective embolisation adopted in the previous studies. A lobar approach aims to destroy previously unidentified occult disease, as well as targeted lesions. All patients had received a minimum of FOLFOX/FOLFIRI as first line treatment, with the majority having failed all second line treatments felt appropriate by a medical oncologist (including Cetuximab and Bevacizumab). A median of 2 embolisations (range 1-5) were performed for each patient with a median dosage of 100mg per embolisation (range 50-200mg) and a median total hepatic exposure of 185mg (range 150-650mg). Adverse event rate after embolisation was 28%, with the majority of events consisting of grade 2 nausea, vomiting and transient liver dysfunction. One patient with large tumour burden (>55% volume replacement) died of MODS after developing delayed hepatic dysfunction 28-days post-embolisation, although it was unclear whether this was due to disease progression or treatment with DEBIRI-TACE. Three-month response rate was 65% (12% complete response, 53% partial response), which reduced to 50% at 6 months and 40% at 12 months suggesting durable disease response. Median overall survival from time of first treatment was 19 months, with an overall progression free survival of 11 months and a hepatic progression free survival of 15 months. These impressive results contrast
favourably with the median survival time for patients who have failed systemic chemotherapy. Eleven patients (20%) had their disease sufficiently downstaged to allow further treatment (Bower et al., 2010), with 4 undergoing resection and 2 undergoing radiofrequency ablation. Evidence of chemotherapy-associated pathological change was not seen in this group. Adverse events were reported in 28%, with an incidence of side-effects greater in those patients who received more than 100mg of the drug, although there was no significant difference in complication rates.

A US led prospective phase I study by the same group is currently recruiting chemo-naïve patients with liver dominant irresectable metastatic colorectal cancer. Patients are treated with FOLFOX +/- DEBIRI-TACE in the off-week of FOLFOX therapy. At a median of 12 cycles of FOLFOX/2 cycles DEBIRI, 4 of 10 patients recruited so far (40%) had been converted to resectability with no evidence of drug induced liver injury in background parenchyma (Martin et al., 2012).

1.11.3.3 Pharmacokinetics of irinotecan after DEBIRI-TACE

Several studies have looked at systemic exposure to irinotecan following DEBIRI-TACE. Eichler et al (Eichler et al., 2012) reported a median maximum irinotecan concentration of 194 ng ml\(^{-1}\) (standard deviation 124) with a maximal concentration achieved 2 hours after treatment. Exposure to SN-38, the active metabolite of irinotecan, was much lower with a maximal concentration of 16.7 ng ml\(^{-1}\) (standard deviation 11.3) achieved after 1 hour.

Martin et al (Martin et al., 2012) reported a peak exposure to irinotecan and SN-38 after 1 hour, with a median dose (range) of 281 (207-484) and 16.4 (11.4-27.8) ng ml\(^{-1}\) irinotecan and SN-38 respectively after first treatment with 100 mg DEBIRI-TACE.
1.12 Irinotecan

Irinotecan (7-ethyl-10[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11, Camptostar) is a camptothecin analogue used in the management of a number of solid tumours (Figure 11). A naturally occurring alkaloid derived from *Camptotheca acuminata*, its anticancer effects were known before its mechanism of action was discovered (Wall et al., 1996). Irinotecan is a prodrug that is converted into the biologically active metabolite SN-38 by cleavage of a dipiperidino side chain (Kawato et al., 1991). SN-38 is 300-200,000 times more cytotoxic than irinotecan (Jansen et al., 1997; van Ark-Otte et al., 1998) but is poorly soluble and highly cytotoxic and so cannot be administered systemically. Only 2-5% of intravenously administered irinotecan is converted to SN-38 (Senter et al., 2001). Animal modeling has suggested that there is no difference in anti-tumour efficacy between i.v. administration of SN-38 or irinotecan (Kawato et al., 1991) suggesting that local activation of irinotecan plays a key role in its anti-tumour effects. Both irinotecan and SN-38 are prone to interconversion between lactone and carboxylate form. The closed lactone configuration is the pharmacologically active form (Hertzberg et al., 1989) with lower pH shifting the equilibrium towards the lactone form. Irinotecan and SN-38 are both inactivated; irinotecan is predominantly detoxified by CYP3A4 to the inactive metabolite APC, whilst SN-38 is conjugated to glucuronic acid by UDP-glucuronoyl transferase UGT1A1.

Wide interpatient variability in the pharmacokinetics and handling of irinotecan have been shown (Mathijssen et al., 2004; de Jong et al., 2004). However, attempts to correlate this with toxicity and drug efficacy have so far failed. Dosing strategies based on body-surface area alone have proved unsuccessful at reducing side-effects (Mathijssen et al., 2002), although a novel dosing algorithm based on
CYP3A4 phenotype improved predictability of the pharmacokinetic profiles between patients (van der Bol et al., 2010).

1.12.1 Topoisomerase 1

The therapeutic target of SN-38 is Topoisomerase 1 (Topo1). When DNA is placed under torsional strain Topo1 binds to and cleaves one strand, allows the intact strand to pass through the DNA-Topo1 complex then rejoins the cleaved DNA strand without damage. SN-38 stabilises the Topo1 DNA complex preventing repair of the single stranded break (Hsiang et al., 1985) resulting in S-phase cell cycle arrest (Hsiang et al., 1989). When a single strand break interacts with a DNA replication fork, the break is converted into a double strand break and cell division stalls. If this break remains unrepaired, the cell is diverted onto a FAS mediated apoptotic pathway (Creemers et al., 1994; Smith et al., 2006; Hsiang et al., 1989; Cao et al., 2010). The effect of Topo1 on cell cycle arrest is therefore more marked in rapidly replicating tumour cells.

Topo1 is highly expressed in around half of colorectal cancers (Boonsong et al., 2002) and it has been suggested that this varying expression may correlate with clinical response to irinotecan with cell modeling demonstrating a clear correlation between cellular levels of Topo1 and sensitivity to irinotecan (Jansen et al., 1997). This hypothesis was assessed in a clinical setting by the UK MRC FOCUS trial, which found that immunohistochemical analysis of CRC identified subpopulations that did or did not benefit from irinotecan containing regimens (Braun et al., 2008). Patients with low Topo1 were found not to benefit from the addition of irinotecan or Oxaliplatin to 5-FU. However, attempts to confirm this finding in an independent cohort (The Dutch Colorectal Cancer Group CAIRO study) failed (Koopman et al., 2009). A large retrospective review by the Hellenic Cooperative Oncology Group (HeCOG) (Kostopoulos et al., 2009) of 498 patients treated with adjuvant therapy
after resection of primary CRC demonstrated that high Topo 1 expression was associated with a reduced risk of death (HR=0.61, 95% CI 0.42-0.88, p=0.01) and those with high Topo1 treated with irinotecan containing regimens had a better OS (HR=0.47, 95% CI 0.23-0.94, p=0.03). Despite these conflicting results, the follow on UK MRC FOCUS 3 trial will attempt to randomize patients with metastatic colorectal cancer based on tumour Topo1 expression and KRAS status. Low Topo1 tumours will be treated with 5-FU, whilst those with high Topo1 will receive 5-FU+/-irinotecan+/-Oxaliplatin with appropriate biologic therapy based on tumour KRAS status. The results of this ambitious study will be extremely informative from both the clinical and personalized treatment perspective.

1.12.2 Metabolism of irinotecan

A variety of different intracellular proteins involved in activation, transport and metabolism of irinotecan allow it to exert its therapeutic effect (Figure 11). Variations in these proteins are thought to contribute to variations in pharmacokinetic profile, and there is now growing interest in these differences as potential predictors of response and toxicity (de Jong et al., 2006).
Figure 11 Schematic diagram showing intracellular metabolism of irinotecan. The key pathways and enzymes are highlighted (key pathway = red arrow, key enzyme = yellow bubble). The water soluble prodrug irinotecan diffuses into cells, where it can be converted to the inactive metabolite APC by CYP3A4 or activated into SN-38 by CES-1 and CES-2. SN-38 acts on Topoisomerase-1, inhibiting DNA repair leading to apoptotic cell death. SN-38 is deactivated by glucuronidation through a UGT1A1 mediated pathway. CES-1=carboxylesterase 1, CES-2=carboxylesterase 2, CYP3A4=Cytochrome p450 3A4, UGT1A1=UDP-glucuronosyltransferase 1A1, ABC=ATP-binding cassette.

1.12.2.1 Carboxylesterase mediated hydrolysis

Mammalian carboxylesterases (CES) are a phase 1 drug metabolism enzyme located in the endoplasmic reticulum and bound by a preserved 4 amino acid C-terminal retention sequence (Morton et al., 1999; Holmes et al., 2010). The most
abundant and well-studied carboxylesterase subtypes in cancer are carboxylesterase (CES) 1 and 2, both of which belong to the β-carboxylesterase family and are involved in the detoxification of xenobiotics and the activation of ester and amide prodrugs. qPCR analysis of 24 primary tumours by Sanghani et al showed that CES-2 was the most abundant carboxylesterase within colorectal cancer (CES-2 ≈ 6 times > CES-1). Interestingly, expression of CES-2 varied ≈ 23 fold between tumour (Sanghani et al., 2003). Tang et al (Tang et al., 2008) found that levels of CES-2 were lower in more advanced colorectal cancers, suggesting that down regulation of this enzyme may be part of a more aggressive phenotype. Using enzyme purifications, CES-2 has been directly demonstrated as the key carboxylesterase involved in the activation of irinotecan in humans (Senter et al., 2001) with a 12.5 time higher affinity and 60-100 time more efficient hydrolysis of irinotecan into SN-38 than CES-1 (Humerickhouse et al., 2000; Sanghani et al., 2003). Despite the relative inefficiency of CES-1 compared to CES-2, it has been suggested that the relative abundance of CES-1 within human liver may mean it is responsible for 50% of hepatic conversion of irinotecan to SN-38 (Hatfield et al., 2011). In vitro attempts to increase tumour levels of CES-2 by recombinant cDNA transfection have shown promise, suggesting that in the future manipulation of tumour phenotype may be possible in order to increase the efficacy of chemotherapy (Matzow et al., 2007).

As well as being expressed in a variety of solid tumours, CES-2 is also expressed in normal tissues (Xu et al., 2002) including small bowel, lung, testes, kidney and liver parenchyma (Guichard et al., 1999). Absolute levels of CES-2 appear highest in hepatic parenchyma, with colorectal primary tumour having levels two to three fold lower (Guichard et al., 1999). Expression of CES-2 within hepatic parenchyma varies widely, with 15 fold interpatient variations reported in some series (Xu et al., 2002). Despite the recognition of a number of single nucleotide polymorphisms in
the CES-2 gene, attempts to correlate a single nucleotide polymorphisms with RNA expression and protein function have failed to identify a clear correlation (Marsh et al., 2004; Bellott et al., 2008).

Surrogate markers of CES activity have been explored. Guemei et al (Guemei et al., 2001) found no association between plasma esterase activity and SN-38 AUC, whilst Shingyoji et al (Shingyoji et al., 2004) found no association between plasma activation of irinotecan and SN-38 AUC. An Italian study measured serum concentrations of irinotecan, SN-38 and SN-38G in 45 patients treated with FOLFIRI. They measured CES-2 mRNA in peripheral blood monocytes as a measure of global CES-2 expression and attempted to correlate this with irinotecan activation, response and toxicity. They identified a high level of variation in expression levels of CES-2 (around 28 fold), which was weakly associated with the irinotecan activation ratio (SN-38_{AUC}+SN-38G_{AUC}/irinotecan_{AUC}). However, when patients were grouped based on median CES-2 expression, those above the median had a statistically significant higher activation ratio than those below with a weaker non-significant association with SN-38_{AUC} (Cecchin et al., 2005).

1.12.2.2 CYP3A metabolism

Multiple plasma, urine, bile and faecal metabolites of irinotecan have been identified (Santos et al., 2000) the most abundant of which is APC, formed by oxidation of the terminal piperidine ring (Smith et al., 2006). NPC, the second most abundant metabolite, is formed from cleavage of the distal piperidine ring and is formed at much lower levels than APC (Santos et al., 2000). Formation of these products is via cytochrome P450 3A subfamily members CYP3A4 and CYP3A5, with the overwhelming majority produced by CYP3A4 (Haaz et al., 1998; Santos et al., 2000). Neither of these metabolites are pharmacologically active (Mathijssen et al.,
Expression of CYP3A4 is lower in colorectal tumour than matched colonic tissue, suggesting that reduced deactivation of irinotecan into APC/NPC may explain the chemo-susceptibility of these tumours (Massaad et al., 1992).

CYP3A is most abundantly expressed in human hepatic parenchyma, where it shows ≈ 30 fold variation in protein and mRNA expression (Watanabe et al., 2004). It has been suggested that genetic polymorphisms in CYP3A may affect function (Marsh et al., 2010). Many polymorphisms in CYP3A coding genes have been identified, but no correlation has been found between genotype and irinotecan metabolism in vitro (Mathijssen et al., 2003; Mathijssen et al., 2004) possibly because these polymorphisms do not change functional enzyme activity in vivo (Xie et al., 2004). However, conflicting data was produced by the recent North American Gastrointestinal Intergroup Trial N9741 which found a clear correlation between the presence of CYP3A5*3C SNP and radiological response to IFL (irinotecan and bolus 5-FU) (29% v 60%; p=0.007) (McLeod et al., 2010).

Concomitant exposure to other drugs and endogenous compounds may alter CYP3A expression levels and therefore change the rate of irinotecan deactivation. For example, Rifampicin and St. John’s Wort activates the pregnane X-receptor (hPXR) increasing expression of CYP3A4 mRNA (Meijerman et al., 2006). Raynal et al (Raynal et al., 2010) found that increased hPXR expression in CRC cells limited chemosensitivity by increasing CYP mediated metabolism. This was validated clinically in a phase I trial which found that patients taking St. John’s Wort treated with irinotecan had a 42% lower circulating dose of SN-38 (Mathijssen et al., 2002).

Phenotypic profiling to guide irinotecan dosing has shown some promise. A randomized control trial gave patients irinotecan dose calculated by CYP3A4 activity (characterized by pre-treatment midazolam clearance) or routine dosing based on body surface area, and demonstrated a reduction in interpatient variation
in irinotecan pharmacokinetics and reduced drug-related toxicity in the phenotypically profiled group (van der Bol et al., 2010).

1.12.2.3 UDP-glucuronosyltransferase

UDP-glucuronosyltransferases (UGTs) represent a key group of enzymes involved in phase II metabolism of irinotecan, converting the active metabolite SN-38 into the inactive glucuronidated form SN-38G. Inhibition of UDP-glucuronosyltransferase by valproic acid prevents 99% of SN-38G formation in rat models whilst UGT induction with phenobarbitol caused a 72% increase in the area under curve (AUC) (Gupta et al., 1997).

Bound to endoplasmic reticulum, UGTs are of major importance in the conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds. The liver represents the major site of glucuronidation, although UGT's are also expressed in a variety of other tissues throughout the gastrointestinal tract (Smith et al., 2006). Considerable variation in the conversion of SN-38 into SN-38G in liver homogenate has been shown in vitro, with a study of microsomes from 25 normal human livers showing 52 fold variation (0.5-26 pmol mg⁻¹ min⁻¹) in activity (Iyer et al., 1998).

Many UGT polymorphisms have been identified, but only a few clinically relevant isoforms have been defined (Guillemette, 2003). UGT1A1, the isoform involved in the conjugation of bilirubin, has been most extensively investigated. UGT1A1*28, which results from a TA dinucleotide insertion into the TATA element of the promoter region, is associated with reduced enzyme production and SN-38 metabolism (Iyer et al., 2002). UGT1A1*28 has an allelic frequency of 39% in Europeans, with a homozygous incidence of 10% (Beutler et al., 1998). This is the same mutation implicated in Gilbert’s syndrome, characterized by an inability to
conjugate bile. A study of 95 colorectal cancer patients treated with irinotecan showed a trend towards worse survival in patients with UGT1A1*28 SNP compared to wild type (p=0.07), and found significantly higher levels of severe diarrhoea in patients homo- or heterozygous for this allele compared to wild type. Haematological impairment followed a similar trend, but did not reach significance (Marcuello et al., 2004). The North American Gastrointestinal Intergroup Trial N9741 (McLeod et al., 2010) performed a retrospective analysis on 520 patients treated with systemic chemotherapy, and found a strong correlation between patients with homozygous mutant UGT1A1*28 treated with irinotecan/Oxaliplatin and grade 4 neutropenia (55% vs. 15%, p=<0.002). A meta-analysis by Hoskins et al (Hoskins et al., 2007) found that the risk of haematological toxicity for patients with UGT1A1*28 SNP was dose-dependent, and suggested toxicity was only relevant at doses above 125mg/m². However, the large UK MRC FOCUS trial using high dose irinotecan (350 mg/m²) showed no correlation between UGT1A1*28 genotype and toxicity (Richman et al., 2009; Seymour et al., 2007). It therefore seems that heterozygosity is less clinically relevant than homozygosity, and UGT1A1*28 genotyping is now recommended by the US FDA prior to commencing irinotecan-based therapies to identify homozygotes (Innocenti et al., 2004). Impaired glucuronidation leading to accumulation of SN-38 may also be expected to increase efficacy of irinotecan therapy. However, a high quality systematic review and meta-analysis assessed whether radiological response to irinotecan based therapy for colorectal cancer was related to UGT1A1 SNPs found no evidence to support this hypothesis (Dias et al., 2012).

1.12.2.4 Adenosine-triphosphate binding cassette (ABC) transporters
ABC transporters are a large family of transmembrane proteins that use ATP to transport molecules across cell membranes (Gottesman et al., 2002) and are classified into 7 distinct families based on the topology of their transmembrane
domains (ABC-A to ABC-G) (de Jong et al., 2006). Irinotecan, SN-38 and SN-38G are actively excreted from cells by these transporters. The biliary recovery of irinotecan was lower in ABCB1 knockout mice, whereas the excretion of SN-38 and SN-38G was unaffected (Iyer et al., 2002). Transfection of KB-3-1 human epidermoid carcinoma cells with ABCC1 cDNA resulted in decreased accumulation and increased resistance to irinotecan, whilst treatment with an ABCC1 inhibitor increased accumulation and restored sensitivity (Chen et al., 1999). Rat modelling with ABCC2 deficient animals also showed reduced irinotecan and SN-38 biliary excretion (Chu et al., 1997; Chu et al., 1997).

ABCG2 SNPs have been studied extensively, with conflicting results. A group of Caucasian patients carrying one defective ABCG2 421A allele were found to have higher serum drug levels (Morisaki et al., 2005) although this hypothesis was rejected by a further larger study (de Jong et al., 2004). In addition, it has been shown that ABCG2 mRNA content of colorectal liver metastases is higher in patients who have been treated with irinotecan suggesting a role for ABCG2 upregulation in acquired chemo-resistance (Candeil et al., 2004).

Irinotecan and its metabolites are also effluxed from cells by other active transport mechanisms including P-glycoprotein multi-drug resistance protein (MDR1), and multi-drug resistance protein-2 (MRP2) although only MDR1 expression appears to correlate with cytotoxicity (Jansen et al., 1998).

1.12.2.5 β-glucuronidase biotransformation

As well as tumour specific-activation of irinotecan through CES, SN-38G reactivation within the tumour microenvironment may be important (Dodds et al., 2002). β-glucuronidase is found in the extracellular matrix surrounding tumour tissue due to lysosomal release from inflammatory cells and necrotic tumour (Bosslet et
al., 1998) and an in vitro analysis of irinotecan metabolism found that at equal concentrations of irinotecan and SN-38G, the rate of β-glucuronidase mediated SN-38 production was higher than that formed directly from irinotecan. However, at pharmacologically relevant levels the rate of production of SN-38 was comparable between the two pathways (Tobin et al., 2006).

Adenovirus mediated transfection of a colorectal cell line to express plasma cell membrane β-glucuronidase increased sensitivity to SN-38G by 80 fold, with animal modeling showing tumours transfected with β-glucuronidase had markedly increased sensitivity to irinotecan. Interestingly, transfection of only 15% of cells led to increased sensitivity suggesting that bystander activation of SN-38G to SN-38 can lead to cell death (Huang et al., 2011).

1.12.3 Transport of irinotecan in blood
In blood, 80% of irinotecan is bound to erythrocytes whereas SN-38 is almost completely bound to albumin and lymphocytes (Mathijssen et al., 2001). In plasma, around 60% of irinotecan is bound to albumin for doses up to 4000 ng/ml, whilst over 94% of SN-38 is bound at doses up to 200 ng/ml. Free irinotecan is metabolized in blood to the active metabolite SN-38 by butyrylcholinesterases, although relative conversion is low (Morton et al., 1999). Intestinal epithelial cells and colon carcinoma cells take up the lactone forms of irinotecan and SN-38 by passive diffusion. The carboxylate form is absorbed by an active pH dependent transport mechanism (Kobayashi et al., 1999). Intracellular conversion of irinotecan into SN-38 within a target cell may not be necessary to achieve a therapeutic effect. Transfection of an A549 colorectal cancer cell line with a recombinant replication-deficient adenovirus vector coding for human carboxylesterase cDNA suppressed cell growth in the presence of irinotecan by 7–17-fold. Diffusion of SN-38 from
neighbouring cells meant only 10% of the A549 cells need to be infected for a cytotoxic effect in 48% of the cells (Kojima et al., 1998).

1.12.4 Pharmacokinetics after intravascular administration of irinotecan

Systemic exposure after IV administration has been thoroughly investigated. Rivory et al demonstrated a maximum systemic concentration of irinotecan of 2.8 µM after infusion with 115 mg m² of irinotecan, with a median peak concentration of 0.08 µM for SN-38 and 1.0 µM for APC (Rivory et al., 1997).

De Jonge et al (de Jonge et al., 2000) reported a $C_{\text{max}}$ for after treatment with 175 mg m² IV irinotecan of 1.98 (+/-0.84) µM, with $C_{\text{max}}$ of 0.075 µM (+/-0.046) for SN-38, 0.46 µM (+/-0.21) for SN-38-G and 0.47 µM (+/-0.13) for APC. $T_{\text{max}}$ for these metabolites was 1.67 hours (+/-0.17), 2.03 hours (+/-0.03) and 2.25 hours (+/-0.25) respectively.

A direct pharmacokinetic comparison of intraarterial and intravenous irinotecan in the same patient demonstrated a peak irinotecan concentration of 8.8 mM and 10.9 mM respectively, with peak SN-38 of 273 nM and 190 nM. (Mambrini et al., 2008)

Intriguingly, the AUC of irinotecan, SN-38 and SN-38G were 5462.2, 87.5 and 904.7 after IA administration, compared to 6799.2, 60.9 and 460.8 for IV infusion, suggesting that intra-arterial administration leads to increased drug activation.

In all of these reports, systemic exposure is significantly higher than that seen after DEBIRI-TACE (See section 1.12.4).

1.12.5 Enterohepatic reactivation of irinotecan

Irinotecan, SN-38 and SN-38G are actively excreted into bile and accumulate within small bowel lumen (Lokiec et al., 1995). Although minor (Atsumi et al., 1995), an additional pathway involves direct transport of irinotecan and its metabolites from
bowel to lumen across the intestinal epithelial cells. When SN-38G is excreted into the intestine, bacterial species including *Escherichia coli*, *Bacteroides* species and *Clostridium perfringens* cleave the glucuronide group from the drug converting it back to SN-38 via the enzyme β-glucuronidase (Takakura et al., 2012). Irinotecan, SN-38 and SN-38G are believed to be reabsorbed to a certain extent by intestinal cells and to enter the entero-hepatic circulation. Accumulation of reactivated SN-38 within the lumen of the bowel is believed to be responsible for irinotecan-associated diarrhea (Kobayashi et al., 1999).

1.12.6 *Metabolism of irinotecan in patients with hepatic disease*

It has been shown that CYP3A4 inhibition in vivo decreases APC formation and increases the systemic dose of SN-38 (Kehrer et al., 2000) with several clinical series reporting increased toxicity in patients with liver disease (Wasserman et al., 1997; Raymond et al., 2002; Schaaf et al., 2006). Caution has therefore been advised in the use of irinotecan in patients with hepatic toxicity.

d'Esposito et al. (d'Esposito et al., 2010) assessed irinotecan biotransformation in patients with normal liver and chronic liver disease. They found that patients with diseased parenchyma had down-regulated CYP3A4 expression and impaired CYP3A4-dependent oxidation of irinotecan into its inactive metabolite APC. They found a similar pattern with carboxylesterase, with impaired CES expression and reduced activation of irinotecan into SN-38. They found that in some cases this change in expression was not equal resulting in altered SN-38: APC ratio, and suggested this may explain reported clinical findings of increased irinotecan toxicity in patients with underlying parenchymal liver disease.
1.13 Aim of thesis

This thesis had two key aims:

• To assess the safety and efficacy of neoadjuvant DEBIRI for patients with CRLM
• To investigate inter-patient variations in treatment response to neoadjuvant DEBIRI

1.14 Plan of study

The plan of study aimed to:

• Assess the safety and efficacy of neoadjuvant DEBIRI through a phase II clinical trial with the following key outcome measures
  o Pathological tumour response (using Blazer score)
  o DEBIRI-related morbidity
  o Surgical morbidity and mortality
  o Radiological response rate (RECIST)
  o Overall and disease free survival
• Develop and validate a novel LC-MS/MS assay in order to measure irinotecan and its key metabolites in
  o Serum
  o Hepatic parenchyma
• Use this assay to measure hepatic drug metabolism using tissue from trial patients, and correlate drug metabolism with pathological tumour response
• Use this assay to measure drug concentrations in serum taken around the time of treatment with DEBIRI, and correlate levels with pathological tumour response
• Perform a global and targeted proteomic analysis of hepatic parenchyma taken from patients treated with DEBIRI and identify proteins associated with individual hepatic activation of irinotecan and pathological tumour response

• To identify potential predictive biomarkers of response to DEBIRI by correlating proteomic data, metabolic profile and pathological tumour response
PARAGON II trial of neoadjuvant DEBIRI for colorectal liver metastases
2.1 Introduction

For patients with irresectable liver metastases, standard of care remains first line systemic chemotherapy with the aim of shrinking tumour and bringing patients to resection. Resectability rates after chemotherapy vary widely, with modern regimens converting up to 60% of patients to resection (Folprecht et al., 2005). Patients who exhibit good pathological response to chemotherapy have better overall survival (Rubbia-Brandt et al., 2007) with tumour replacement by fibrosis associated with good long term outcome (Poultsides et al., 2012). Response to chemotherapy is also known to correlate with resection rate (Folprecht et al., 2005) and so it is vital that patients are treated with the most effective chemotherapeutic regimen possible.

The benefit of perioperative therapy for initially resectable disease is less clear. A large multicentre study (EORTC 40983) compared long term outcomes following 12 cycles of perioperative FOLFOX (6 before surgery, 6 after) against surgery alone for initially resectable disease (Nordlinger et al., 2008). Three year PFS was 36.2% in the perioperative chemotherapy arm compared with 28.1% in the surgery alone group but this produced no improvement in long term overall survival (Nordlinger et al., 2012). Growing evidence suggests that preoperative chemotherapy is associated with agent-specific patterns of chemotherapy-associated liver injury (CALI) (Parikh et al., 2003; Fernandez et al., 2005; Rubbia-Brandt et al., 2004), with Vauthey et al (Vauthey et al., 2006) reporting a clear association between CALI and 90-day post-operative mortality. The decision to treat resectable disease with neoadjuvant therapy is therefore a delicate balance between risk and benefit, with little way of predicting which patients are likely to benefit most.

The liver dominant pattern of metastatic colorectal cancer has led to the development of liver-directed therapy in an effort to increase metastatic
exposure whilst reducing off-target side-effects. Drug eluting beads are compressible polyvinyl alcohol (PVA) microspheres loaded with irinotecan (DEBIRI), and have shown impressive long-term outcomes in heavily pre-treated irresectable patients (Martin et al., 2010). However, there is no evidence that it is safe to perform hepatic surgery on patients who have been treated with DEBIRI-TACE. The demonstration of safety in this setting is vital before further trials assessing the use of DEBIRI-TACE in a downsizing/conversion or true neoadjuvant protocol. The primary objective of this study was therefore to evaluate resectability after neoadjuvant DEBIRI-TACE for liver metastases from colorectal cancer.

2.2 Methods

2.2.1 Study Design

This was a pan-European multicentre open label, single arm phase II study. The trial had full ethical approval (South West Regional Ethics Committee reference 08/H0206/51, see appendix 3) and was registered at clinicaltrials.gov (NCT00844233).

2.2.2 Patient identification

Patients were identified as eligible for recruitment at a regional specialist hepatobiliary MDT. The trial had a recruitment target of 40 patients successfully treated with DEBIRI-TACE.

2.2.3 Inclusion criteria

Patients were aged between 18-80 years, with an ECOG status ≤2 i.e. symptomatic but ambulatory >50% of waking hours or better functional status.
All patients had undergone, or were deemed suitable for, an R0 primary colorectal resection and had liver-dominant metastatic disease with a maximum of 4 potentially resectable colorectal liver metastases (initially only unilobar distribution was included, but this was later expanded to include bilobar disease). Resectability was defined by the local hepatobiliary MDT after full staging with triple-phase CT chest/abdomen/pelvis, MRI with liver-specific contrast and PET-CT. Previous systemic chemotherapy within 1 month of recruitment was not allowed, nor was any previous exposure to irinotecan.

2.2.4 Exclusion criteria

Exclusion criteria included peripheral neuropathy, uncontrolled cardiac failure or angina pectoris, hypertension or arrhythmia. Patients with abnormal WCC, platelets or renal function were excluded, as were those with a contraindication to irinotecan treatment and concomitant use of CYP3A4 inducers e.g. St John's Wort. Contraindications to transarterial chemoembolisation included allergy to contrast media, porto-systemic shunt, hepatofugal blood flow and severe peripheral atherosclerosis.

2.2.5 Study endpoints

2.2.5.1 Primary endpoint
2.2.5.1.1 Tumour resectability after neoadjuvant DEBIRI-TACE

The trial had a primary endpoint of R0 tumour resectability after DEBIRI-TACE. R0 resection was defined as a negative margin of ≥2mm on histopathological assessment for lesions treated with DEBIRI.
2.2.5.2 Secondary endpoints

2.2.5.2.1 Safety of DEBIRI-TACE

All observed toxicities and side effects after delivery of DEBIRI-TACE were graded according to NCI Common Terminology Criteria for Adverse Events (NCI CTCAE v 3.0) (Trotti et al., 2003).

2.2.5.2.2 Radiological assessment of tumour response

Triple-phase contrast enhanced CT was performed within 4 weeks of trial recruitment as a baseline assessment, then repeated 4 weeks after DEBIRI treatment immediately prior to resection. Two independent reviewers performed assessment of radiological response. Response of treated lesions were assessed using RECIST criteria v 1.1 (Eisenhauer et al., 2009). In brief, complete disappearance of all lesions was considered complete response, >30% reduction in sum of longest diameter of target lesion was classified as partial response, >20% increase in the sum of the longest diameter of target tumour was considered progressive disease. Stable disease was defined as fulfilling neither of the criteria for partial response nor progressive disease. The appearance of new lesions was considered as progressive disease. Response was also evaluated using novel morphological criteria validated by the MD Anderson group (Chun et al., 2009). Briefly, each lesion was assigned to one of three groups. Group 1 was characterized by homogenous low attenuation with a thin, sharply defined tumour-liver border. Group 3 lesions were characterized by heterogeneous attenuation and a thick poorly defined liver tumour interface. Group 2 included tumours that were neither group 1 nor group 3. Morphological response were defined as optimal if the lesion changed from group 3 or 2 to 1, incomplete if 3 to 2, and none if the lesion did not change appearance. Where more than one lesion was present, morphological
response was defined as that seen in the majority of lesions. The appearance of new disease between imaging was defined as progressive disease for all criteria.

After resection, follow-up CT scans of chest, abdomen and pelvis were performed at 3, 6, 9 and 12 months.

2.2.5.2.3 Pathological assessment of tumour

Immediately after resection, tumour was placed in formalin and processed according to normal hospital protocol. Timing of specimen cut-up after placement in formalin was not recorded or controlled, but was consistently less than 48 hours after delivery. Gross pathological examination of the specimen was then performed. Histopathological samples were prepared by embedding tissue in paraffin according to standard hospital procedures. Confirmation of adenocarcinoma was made using morphological assessment. The sampling of the tumour for histopathological evaluation was extensive, and included the periphery of the tumour including adjacent normal liver, the tumour and resection margin, and sampling of the tumour with a map of the tissue blocks. The location of each tissue sample was identified as either centre of tumour, mid part of tumour, periphery of tumour, tumour and adjacent liver parenchyma, or tumour and resection margin (see Figure 12). Untreated lesions detected incidentally at the time of resection were not included in pathological analysis as part of the trial protocol.
**Figure 12** Tumour sampling strategy. Paraffin embedded blocks were produced from tissue taken from the centre of the tumour (1), mid-part of the tumour (2), periphery of the tumour (3), the border between tumour periphery and surrounding hepatic parenchyma (4) and the tumour and resection margin (5).

Assessment of these tissue blocks was by microscopic examination. Background hepatic parenchyma was assessed and degree of steatosis and fibrosis quantified using a validated method (Kleiner et al., 2005). In brief, the score was defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2) with a total score ranging from 0 to 8 (See table 8). Patients with a score $\geq 5$ were considered to have steatohepatitis.
<table>
<thead>
<tr>
<th>Pathology</th>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>&lt;5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5%-33%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt;33-66%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;66%</td>
<td>3</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>No foci</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt;2 foci per x 200 field</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2-4 foci per x 200 field</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;4 foci per x 200 field</td>
<td>3</td>
</tr>
<tr>
<td>Ballooning</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Few balloon cells</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Many cells/prominent</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 8 Summary of Kleiner score (Kleiner et al., 2005) for steatohepatitis in human hepatic parenchyma. Points are given for type and degree of pathological change, with a cumulative total from 0-8. A score ≥5 is considered pathognomonic of steatohepatitis.

Tumour was assessed and degree of differentiation, involvement of tumour border, and vascular invasion (defined as cancer cells identified within an epithelial lined channel) was commented on. Microscopic examination of resection margin was also performed.

Assessment of degree of pathological response was made using a minimum of 4 blocks stained with haematoxylin-eosin by a specialised gastrointestinal pathologist. This was performed using a validated method described by Blazer et al (Blazer et al., 2008), whereby an estimation of proportion of lesion consisting of viable tumour cells, proportion consisting of fibrotic tissue and proportion consisting of necrosis within the whole tumour assessed across all 4 blocks was made. Complete pathological response was defined as no viable tumour cells, major response as 1%-49% of viable tumour cells and minor response as >50% viable tumour cells. Patients with no response (100% viable tumour) were included in the minor response group.
2.2.5.2.4 KRAS/BRAF genotyping

All KRAS and BRAF testing was performed by ManGen (Central Manchester University Hospitals), a commercial UKNEQAS compliant facility.

Ten 10µm thick sections of FFPE primary colorectal cancer were collected and placed in a 1.5mL Eppendorf tube. The tissue sample was selected to maximise tumour cell content, with a minimum of 50% tumour tissue. DNA was extracted using a QIAamp DNA FFPE Tissue Kit and quantified by spectrophotometry using a NanoDrop ND-100 (Thermo Fisher Scientific). KRAS mutations (p.G12A, p.G12V, p.G12R, p.G12C, p.G12D, p.G12S and p.G13D) were analysed with TaqMan probes (Applied Biosystems) using a validated PCR method. Samples testing negative for KRAS mutation were further characterised for BRAF p.V600E mutations using a similar approach.

2.2.5.2.5 Recurrence following resection

Recurrence was defined as the identification of any new disease compared to the clinical and radiological assessment performed immediately prior to surgery. Time to recurrence was defined as the time from surgery to radiological confirmation of recurrent disease (See section 2.2.6.4).

2.2.6 Study schedule

2.2.6.1 Baseline assessment

After obtaining written informed consent to trial participation, patients underwent baseline assessment within 4 weeks of proposed chemoembolisation date. A triple-phase CT chest/abdomen/pelvis was required prior to embolisation to provide a baseline for assessing radiological response.
Portal vein patency was formally assessed using this CT. Pre- and post-operative CEA levels were not routinely recorded.

2.2.6.2 Neoadjuvant chemoembolisation

Chemoembolisation with DEBIRI was performed within 4 weeks of baseline screening visit and 4 weeks prior to the planned resection. Treatment consisted of a nominal dose of 2ml of DC Bead of 100 to 300 μM diameter containing 200 mg of irinotecan (PARAGON Bead®). The beads were hydrated with water for injection, and mixed with a non-ionic contrast media in the vial immediately prior to use according to the manufacturers instructions.

Using a unilateral femoral approach, selective catheterisation of the hepatic artery was performed. Vascular access was obtained via the common femoral artery and a guidewire advanced under fluoroscopic guidance. A 5 Fr. sheath was then inserted over the guidewire. The superior mesenteric artery was selected and an angiogram performed to identify any aberrant arterial anatomy and verify portal patency. The coeliac axis was selected and an angiogram completed. The catheter and guidewire were used to select the proper hepatic artery and a limited angiogram performed to identify the branches of the hepatic artery. Depending on the location of the lesions to be treated, the right or left hepatic artery was selected distal to the cystic artery (if visualised). Initially very selective embolisation was performed with bead delivery direct to the subsegment containing the tumour. As experience grew, a more proximal catheter placement into the right or left hepatic artery was used.

Once catheter placement was confirmed, the embolic mixture was injected slowly into the artery supplying the area where the lesion was located until the blood flow became sluggish i.e. embolisation did not proceed to full stasis. If lesions were located in more than one segment, embolic mixture was injected
into the arteries feeding the individual segments where the tumours were located. If the embolisation endpoint was achieved before the delivery of 2ml irinotecan Bead, the injection was stopped and the volume of beads administered was recorded.

In prior studies of chemoembolisation with DC Bead, patients reported moderate to severe post procedural pain (Fiorentini et al., 2008). An aggressive medication strategy was adopted to manage this; 1g paracetamol, 50mg Diclofenac and a 10mg IV bolus of morphine was given at the time of embolisation, followed by regular paracetamol and on-demand opiate analgesia (PCA pump). Intra-arterial injection of 2-4ml of lidocaine (1%) prior to injection of DC Bead has previously been shown to reduce peri-procedural pain and was therefore used during this study (Fiorentini et al., 2008).

Blood samples were taken pre-TACE, then at 1 and 6-hours post-TACE for drug and metabolite analysis (See section 4.2.2).

2.2.6.3 Surgery

Approximately four weeks after embolisation, patients underwent repeat CT chest/abdomen/pelvis to assess radiological response to DEBIRI followed by surgical resection. All patients underwent resection during open surgery by an experienced hepatobiliary surgeon. Laparotomy was performed and extrahepatic abdominal disease excluded by full inspection. Intraoperative ultrasound (IOUS) was routinely performed to guide surgical planning. If the size of metastases reduced significantly after chemoembolisation, the extent of liver resection was not modified and therefore still performed as initially planned. However, when previously undetected deposits were discovered (either during surgery or during peri-surgical imaging), resection was adapted to ensure the removal of all identifiable disease. Low volume anaesthesia was
used aiming for a central venous pressure (CVP) of or below 5 mm Hg. Liver parenchyma was transected with the Cavitron ultrasonic surgical aspirator (CUSA, Valleylab). Use of intermittent vascular inflow occlusion was at the discretion of the operating surgeon. A curative resection of the metastases with a clearance of ≥1 cm of normal parenchyma was the surgical aim. However, metastases were considered resectable with a clearance less than 1 cm provided complete macroscopic resection of disease was possible.

As soon as the specimen was delivered from the patient, samples of normal hepatic parenchyma and tumour were taken and immediately snap frozen for further analysis. Blood samples were also taken for drug and metabolite analysis immediately before the start of surgery (See section 4.2.2).

Patients were managed post-operatively in a critical care or ward environment based on qualitative anaesthetic assessment at the end of surgery. Post-operative morbidity was routinely recorded using the Clavien-Dindo classification (Dindo et al., 2004).

2.2.6.4 Follow-up visits

Follow-up visits took place 4-6 weeks after surgery to assess wound healing and recovery, as is routine after major resections. There was further outpatient review at 3, 6, 9 and 12 months post-resection, followed by routine 6-monthly review. These follow-up visits included clinical examination, liver function tests and a triple-phase CT chest/abdomen/pelvis to detect disease recurrence.

2.2.6.5 Statistical analysis

Patients were stratified according to pathological response rates. For patients with more than one lesion, the mean response score for all targeted lesions
was used for stratification. This was calculated by the sum of % viable tumour in all lesions, divided by the number of resected lesions. Quantitative and qualitative variables were expressed as medians (with range) and frequencies. Comparisons between groups were analysed with the chi-square test or Fisher exact test for proportions and the Mann-Whitney U test for continuous variables.

Overall and disease free survival were calculated from the date of liver resection to the date of last follow-up, recurrence or death using the Kaplan-Meier method. Comparisons were made using log-rank test.

To identify factors associated with OS and DFS in the entire cohort, the following variables were assessed using univariate analysis; sex (male vs. female), age (>65, <65 – median age of patient cohort), presentation (synchronous (<6 months) vs. metachronous), interval between primary colorectal resection and liver resection (<25 months, >25 months), size of lesion (<25, >25 mm longest diameter), margin (R0 vs. R1), pathological response to chemotherapy (major vs. minor, complete vs. minor), number of lesions (1, >1) and KRAS status (Mutant vs. wild-type). All variables associated with P<0.05 in the univariate proportional hazards model were entered into a cox proportional hazards multivariate model using a forward step wise procedure. P <0.05 was considered significant. All statistical analyses were performed using IBM SPSS Statistics (v.20).
2.3 Results

2.3.1 Patient demographics

Forty-nine patients were recruited to the study. Forty underwent DEBIRI-TACE. Reasons for not proceeding to TACE included consent withdrawal (n=2), bilobar disease (n=2, beginning of protocol when bilobar disease was considered an exclusion criteria), tumour involving gallbladder wall (n=1), suspected hepatocellular carcinoma (n=1), arterial access difficulty (n=2) and contrast medium allergy (n=1).

KRAS/BRAF status was determined in 28 local patients where tissue was accessible. The 8 patients with KRAS mutant tumours had KRAS G12A (n=2), G12D (n=1), G12V (n=2), G12C (n=2) and Q61H (n=1) mutations. Three further patients did not have enough tumour left after treatment for tissue typing.

Patient demographics are detailed in table 9.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Median age, years (range)</th>
<th>Sex M:F</th>
<th>Median ECOG (range)</th>
<th>Synchronous:metachronous</th>
<th>Median interval between primary surgery and diagnosis of metastases, months (range)</th>
<th>Median number of lesions per patient (range)</th>
<th>Median maximal diameter of lesion, mm (range)</th>
<th>KRAS wild type (n=28)</th>
<th>BRAF wild type (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65 (39-78)</td>
<td>25:15</td>
<td>0 (0)</td>
<td>11:29</td>
<td>25 (10-93)</td>
<td>1 (1-4)</td>
<td>21 (4-150)</td>
<td>20 (71%)</td>
<td>28 (100%)</td>
</tr>
</tbody>
</table>

Table 9 Demographic details of patients undergoing treatment with DEBIRI-TACE.
2.3.2 **DEBIRI-TACE**

All 40 patients underwent uneventful DEBIRI-TACE. Sixty-six discrete lesions were targeted. Patients received a median dose of 103 mg irinotecan (range 64-175 mg). No patients required sedation, and all patients were discharged home within 24 hours, apart from one who remained in hospital for an extra 24 hours because of social reasons.

One patient developed post-DEBIRI pancreatitis (CTCAE grade 2, treated with supportive care) and was readmitted after 48 hours (2.5% morbidity). After an uneventful 3-day inpatient stay he was discharged home, and underwent liver resection 95 days after TACE. No patients developed any systemic toxicity to irinotecan.

2.3.3 **Surgery**

All forty patients proceeded to surgery. Median interval from DEBIRI-TACE to surgery was 30 days (range 22-95). At laparotomy, 2 patients were found to have unresectable disseminated peritoneal disease (one with omental seedings, one with multiple nodules on small bowel mesentery) neither of which were identified on preoperative CT or PET imaging. Thirty-eight patients therefore underwent liver resection (95% resection rate). Twelve patients (32%) underwent anatomical resection (7 left hemihepatectomy, 4 left lateral sectionectomy, 1 right hemihepatectomy), with the remainder undergoing non-anatomical metastectomy. Vascular exclusion time was less than 30 minutes in all cases.

Four previously undetected intrahepatic lesions were found in three patients (See section 2.3.4.3). Sixty-eight lesions were therefore resected. Fifty were R0 (>2mm) resections (R0 resection rate 74%) according to trial protocol (see section (See section 2.2.5.1), with the remaining 18 undergoing R1 resection.
(R1 resection rate 26%). If R0 margin was defined as ≥1mm, 84% of lesions were resected with R0 margin.

Two patients died within 30 days of surgery (30-day mortality 5%), neither of which was TACE related. One patient died on the day of surgery from haemopneumomediastinum following insertion of a central line on the critical care unit. The second patient died 8 days after surgery, having developed aspiration pneumonia followed by multi-organ dysfunction syndrome (MODS).

Eleven patients (27.5%) developed complications (Dindo-Clavien grade 1-4), with 2 (5%) developing grade 3 or 4 complications i.e. requiring intervention (one small bile leak requiring percutaneous drainage, one post-operative wound infection requiring clip removal).

2.3.4 Pathological response

One patient was found to have hepatocellular carcinoma on histopathology and so was excluded from further pathological and long-term analysis. The 4 untreated lesions were outside the trial protocol, and so were not included for analysis. The remaining 63 treated lesions were assessed for pathological response. Median tumour diameter was 21mm (4-150 mm). Median viable tumour was 20% (range 0-100), necrosis 50% (range 0-100) and fibrosis 17% (range 0 -70). Tumour response was defined as complete in 11 lesions (17%), major in 37 lesions (59%), minor in 14 lesions (22%) and no response in 1 lesion (2%) (Figure 14).
Figure 13 Representative photomicrographs of pathological tumour response after treatment with DEBIRI. * = tumour, < = tissue fibrosis, # = normal hepatic parenchyma. The black arrow shows distorted tissue architecture with parenchymal replacement with fibrosis at the site of a previous lesion. Response was classified by global assessment of at least 4 tumour blocks, with an estimation made of proportion of viable tumour remaining. (A) Minor response >50% tumour remaining, (B) Major response 1-49% tumour remaining, (C) Complete response 0% tumour remaining. All images x 10 magnification.

Figure 14 Column chart showing pathological response grade of colorectal liver metastases treated with DEBIRI. Complete response = 0% viable tumour, major response = 1-49% viable tumour, minor response = >50% viable tumour.
2.3.4.1 *Steatosis and steatohepatitis after DEBIRI-TACE*

There was little evidence of steatosis or steatohepatitis in any patients treated with DEBIRI-TACE. Median Kleiner score was 0 (range 0-5), with 76.3% having a score of 0. Only one patient (2.6%) had a score (5) diagnostic for steatohepatitis (Figure 15). There was no quantifiable difference between tissue immediately adjacent to treated tumour and tissue away from the treatment zone in any patients.

![Column chart showing Kleiner score for steatosis, steatohepatitis and fibrosis in background hepatic parenchyma from patients treated with DEBIRI. Median Kleiner score was 0, with 76.3% of patients having a score of 0 (no evidence of steatosis or steatohepatitis). Only one patient (2.6%) had a score ≥5, diagnostic of steatohepatitis.](image-url)
2.3.4.2 Comparison of patient characteristics by pathological response rate

Thirty-seven patients who underwent resection for CRLM were grouped by pathological response. Baseline characteristics are described in table 10. All 3 groups were similar, with a marginally but statistically non-significant increased number of lesions in the complete response group ($p=0.07$). The major response group had a significantly larger median tumour size than the minor ($p=0.04$) and complete ($p=0.03$) response groups.
Table 10 Characteristics of patients who underwent hepatectomy for CRLM after neoadjuvant DEBIRI stratified by pathological tumour response. All 3 groups were similar, although median lesion size was significantly higher in the major response group compared to the minor and complete response group ($l$=Minor vs. major, $x$=Major vs. complete).
2.3.4.3  Comparison of treated and untreated lesions in the same patient

Three patients had intrahepatic disease that was not identified preoperatively and so was not treated with DEBIRI-TACE. This allowed the comparison of treated and untreated lesions from the same patient, enabling assessment of pathological response directly attributable to DEBIRI-TACE.

2.3.4.3.1  Patient 1

Pre-treatment imaging identified a lesion in segment VIII. Angiography at the time of embolisation confirmed a hypovascular lesion in segment VIII, and irinotecan bead was administered via the segment VIII segmental artery. At laparotomy, IOUS demonstrated the previously identified and treated lesion in segment VIII as well as a second untreated lesion in segment IV. The treated lesion in segment VIII had a longest diameter 25mm, and showed no residual tumour and 100% replacement with necrotic tissue. By contrast, the untreated lesion in segment IV (longest diameter 25mm) demonstrated 30% residual tumour, 40% necrosis, and 30% fibrosis (Figure 16).
Figure 16 Photomicrograph (x20) of (A) targeted and (B) non-targeted CRLM from the same patient treated with DEBIRI. The treated lesion shows an absence of viable tumour (*), with complete replacement with necrotic tissue (♯) surrounded by normal hepatic parenchyma (=). The irinotecan beads (arrow) are clearly visible within the vasculature. By contrast, the untreated lesion shows islands of viable cells with minimal necrosis and fibrosis. Magnification x 10.

2.3.4.3.2 Patient 2

The patient was found to have a single lesion supplied by segment VI artery, which was treated by superselective (subsegmental) embolisation. At laparotomy IOUS demonstrated the previously identified and treated lesion in segment VI as well as a second previously unidentified lesion in the same segment, both of which were easily resected. The pre-operatively identified lesion in segment VI had a longest diameter of 15mm, with no evidence of viable tumour, 60% necrosis and 40% fibrosis. By contrast, the untreated lesion
in segment VI (longest diameter 14 mm) demonstrated 60% residual tumour, 30% necrosis, and 10% fibrosis.

2.3.4.3.3 Patient 3

This patient was found to have a lesion on the border of segment II and IVa with longest diameter of 17mm. The patient underwent embolisation in a lobar fashion via the left hepatic artery until partial occlusion of the left subsegmental arteries was achieved. Pre-operative CT demonstrated a reduction in diameter of the target lesion to 10mm (a 41.2% reduction, RECIST partial response). However, on this imaging a second tumour in segment II (within the lobar treatment zone) could now clearly be identified. Radiological assessment suggested this lesion was 100% necrotic. Retrospective review of the initial staging imaging showed no evidence of this lesion prior to treatment with irinotecan bead. During surgery, IOUS detected two further lesions. One, in segment III, had been treated by lobar infusion. The second intraoperatively detected lesion was in segment VII and had not been exposed to irinotecan bead. Post-operative examination of all 4 resected tumours showed varying degrees of response. All three lesions in the left hemiliver that had been treated with irinotecan bead (including the original target lesion in segment II/IVa, the second inadvertently treated lesion in segment II only identified after treatment with irinotecan bead but before resection, and the intraoperatively identified and inadvertently treated lesion in segment III) demonstrated absence of viable tumour and 100% replacement with fibrotic or necrotic tissue. By contrast, the lesion in segment VII that was not in the treatment zone for irinotecan bead demonstrated 45% residual tumour, 50% necrosis and 5% fibrosis.
2.3.5 Radiological response

Radiological response immediately prior to resection was evaluable in 22 patients with 37 discrete lesions (Table 11). In the 10 patients with multiple metastases, response criteria were the same for all lesions. Inter-observer agreement on radiological response was high, with 100% agreement on RECIST criteria. Inter-observer agreement was also high for morphological response, with agreement in 84% of cases ($\kappa=0.82$). All disagreement was solved by consensus review.

<table>
<thead>
<tr>
<th></th>
<th>No. patients (%) (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median, range yrs.)</td>
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<td>6 (27)</td>
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<tr>
<td>Colonic primary</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Solitary liver metastasis</td>
<td>12 (55)</td>
</tr>
<tr>
<td>Multiple liver metastases</td>
<td>10 (45)</td>
</tr>
<tr>
<td>Median tumour size (longest diameter mm, range)</td>
<td>24 (4-150)</td>
</tr>
<tr>
<td>Interval from DEBIRI to resection (median, range days)</td>
<td>32 (27-95)</td>
</tr>
<tr>
<td>Interval from DEBIRI to preoperative imaging (median, range days)</td>
<td>33 (16-98)</td>
</tr>
</tbody>
</table>

Table 11 Demographics of patients assessed for radiological response

2.3.5.1 Radiological response and pathological tumour response

There was no correlation between radiological response rates and pathological tumour response. Twenty of 22 patients had stable disease by RECIST criteria, with the remaining 2 demonstrating progressive disease. Interestingly, both patients with progressive disease had a major pathological response with <10% viable tumour on post-resection analysis.

Eleven patients demonstrated incomplete morphological response, with 11 showing no response (Figure 17). Morphological response showed no association with degree of residual tumour: lesions showing no morphological
response had a median of 10% viable tumour (IQR 0-40), compared to 23% for partial response (IQR 10-30) (p=0.79). There was no association between morphological response and amount of tumour necrosis, with non-responders having a median of 40% necrosis (IQR 0.33-0.75), compared to 55% for partial responders (IQR 0.38-0.73) (p=0.55). Non-responsive lesions had a median 10% fibrosis (IQR 10-40), compared to 13% (IQR 10-33) for partial responders (P=0.91) (Figure 18).

When pathological response was stratified as major, minor or complete, 10 of 17 (59%) patients with complete/major pathological response had an incomplete morphological response.
Figure 17 CT scan of patient [A] before and [B] 34 days after single treatment with DEBIRI. The lesion shows >20% increase in diameter (progressive disease using RECIST). This lesion had 10% viable tumour on post-resection histopathological analysis. CT scan of patient [C] before and [D] 29 days after treatment. Enhancement of the lesion became less heterogeneous with a more clearly defined tumour-liver interface. This was defined as an incomplete morphological response. This lesion had 20% viable tumour on post-resection histopathological analysis.
Figure 18 Box and whisker plot showing correlation between morphological response and pathological tumour assessment. Morphological response did not correlate with amount of viable tumour (A), degree of fibrosis (B) or necrosis (C). The dark line inside the box represents the median value, whilst the end points of the whiskers represent minimum and maximum values. Lower and upper edges of boxes represent 25th and 75th percentiles. P-value by Mann Whitney U test.
2.3.6  Long-term outcome

2.3.6.1  Overall survival

Survival analysis was performed on 35 of 38 patients who underwent hepatectomy for CRLM (2 early post-operative deaths were excluded from analysis, as was one patient found to have hepatocellular carcinoma on histopathology). At a median follow-up of 313 days (range 63-565) one patient had died of cancer progression with widespread hepatic and pulmonary metastases 565 days after resection. Nominal 1-year overall survival was 100%.

2.3.6.2  Disease free survival

There were 15 recurrences detected with a median DFS of 379 days (95% CI 276-498) (Figure 19). Nominal 1-year DFS was 56%. Nine patients had intrahepatic recurrence, four had hepatic and pulmonary recurrence whilst two had only pulmonary recurrence.
Figure 19 Kaplan-Meier curve showing disease free survival after hepatic resection following neoadjuvant DEBIRI-TACE. Median DFS was 379 days. The table shows number at risk for each 100-day time interval.

<table>
<thead>
<tr>
<th>Days</th>
<th>Number at risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>100</td>
<td>31</td>
</tr>
<tr>
<td>200</td>
<td>18</td>
</tr>
<tr>
<td>300</td>
<td>13</td>
</tr>
<tr>
<td>400</td>
<td>6</td>
</tr>
</tbody>
</table>

2.3.7 Predictors of long term outcome

The results of univariate and multivariate analyses of factors associated with DFS are summarized in Table 12.

On univariate analysis, synchronous presentation (p=0.03) and KRAS status (p=0.04) were associated with a poor DFS (Figure 20). On multivariate analysis, only KRAS status was independently associated with DFS (HR=0.12, CI 0.02-0.73, p=0.02).
<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=35 (%)</td>
<td>Median DFS (days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number progressed</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
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<td>13</td>
</tr>
<tr>
<td></td>
<td>&gt;65</td>
<td>22</td>
</tr>
<tr>
<td>Presentation</td>
<td>Synchronous</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Metachronous</td>
<td>26</td>
</tr>
<tr>
<td>Interval from</td>
<td>primary to secondary</td>
<td>&lt;25 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;25 months</td>
</tr>
<tr>
<td>Days between</td>
<td>TACE and resection</td>
<td>&lt;30 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;30 days</td>
</tr>
<tr>
<td>Size, mm</td>
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<td>17</td>
</tr>
<tr>
<td></td>
<td>&gt;25</td>
<td>18</td>
</tr>
<tr>
<td>Margin</td>
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<td>26</td>
</tr>
<tr>
<td></td>
<td>R1</td>
<td>9</td>
</tr>
<tr>
<td>Pathological</td>
<td>Minor</td>
<td>6</td>
</tr>
<tr>
<td>response</td>
<td>Major</td>
<td>25</td>
</tr>
<tr>
<td>Pathological</td>
<td>Complete</td>
<td>4</td>
</tr>
<tr>
<td>response</td>
<td>Vascular invasion</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>24</td>
</tr>
<tr>
<td>KRAS status</td>
<td>Mutant</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Wild-type</td>
<td>20</td>
</tr>
<tr>
<td>Number of lesions</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&gt;1</td>
<td>15</td>
</tr>
<tr>
<td>RECIST response</td>
<td>SD</td>
<td>20</td>
</tr>
<tr>
<td>@ 4 weeks</td>
<td>PD</td>
<td>2</td>
</tr>
<tr>
<td>Morphological</td>
<td>None</td>
<td>11</td>
</tr>
<tr>
<td>response @ 4</td>
<td>Incomplete</td>
<td>11</td>
</tr>
<tr>
<td>weeks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 12 Univariate and multivariate analysis of clinicopathological variables associated with DFS in 36 patients who underwent hepatectomy following neoadjuvant DEBIRI.
Figure 20 Kaplan-Meier curve showing the negative prognostic effect of KRAS mutation on disease free survival after neoadjuvant treatment with DEBIRI-TACE (log-rank p=0.03). The table shows number at risk for each 100-day time interval.
2.4 Discussion

This study clearly demonstrates the safety and efficacy of single treatment neoadjuvant DEBIRI-TACE prior to liver resection for colorectal liver metastases.

One of the theoretical advantages of DEBIRI-TACE is its targeted nature and ease of administration, which may transfer to reduced treatment costs. Ninety-eight percent of patients in the current study were discharged within 24 hours of embolisation. Although not fulfilling the definition of a true “day case” intervention, DEBIRI-TACE was performed quickly and easily with short inpatient stay and low complication rates (2.5% adverse event rate). Previously, a US-based registry of patients with heavily pretreated liver dominant colorectal cancer reported a 28% adverse event rate, with a bead specific complication rate of 1% (Martin et al., 2010), perhaps reflecting the pretreated nature of the patient cohort as well as higher tumour burden. In both series the bead specific complication rate compares favourably with the toxicity (Kemeny et al., 2009) and technical complication rates (Allen et al., 2005) reported after liver directed therapy delivered by hepatic arterial infusion pumps.

Surgical safety was not compromised by neoadjuvant treatment with DEBIRI-TACE. Hepatic resection was performed in thirty-eight of forty cases (resectability rate 95%), with two patients having widespread intraperitoneal carcinomatosis discovered at laparotomy. This pattern of disease is difficult to detect on preoperative imaging, and the delay of 4 weeks between DEBIRI-TACE and surgery is unlikely to be sufficient interval for the development of micrometastases. 74% of lesions treated with DEBIRI-TACE were resected with an R0 margin, with 100% R0/R1 resection. The R0 resection rate is slightly higher than other reported series, and likely reflects the easily resectable nature of patients recruited to the study.
The trial protocol defined an R0 resection as a margin of at least 2mm, whilst the International Union Against Cancer (UICC) defines R0 and R1 resections as the absence (tumor-free margin >=1 mm for all detected lesions) or presence (tumor-free margin 0 mm) of microscopic tumor invasion of the resection margins (Wittekind et al., 2002). Adopting the UICC definition increased the R0 lesional resection rate to 84%. Beyond 1mm (i.e. microscopically negative margin) width of resection margin does not appear to impact on long-term survival (Pawlik et al., 2005). This study confirmed these findings, with patients undergoing R0/R1 resection margin having equivalent disease free survival.

Post-operative surgical mortality (5%) was higher than other contemporary series (Nordlinger et al., 2008; Adam et al., 2008; Vauthey et al., 2006), which report 30-day mortality for hepatectomy after neoadjuvant chemotherapy consistently less than 2%. However, it seems unlikely that either of the deaths seen in this study were directly related to the neoadjuvant administration of DEBIRI-TACE. One patient died from anaesthetic misadventure, whilst a second patient died from a recognised postoperative complication. Post-operative morbidity was in keeping with other published series (Dunne et al., 2014).

The pathological response rates demonstrated in the treated lesions are impressive, with 76% of lesions showing a major or complete pathological response. The intraoperative identification of untreated lesions in a treated patient gives an interesting control group. In patient 1, two distinct lesions were resected; one from within the treated segment, and one from an untreated segment. The difference in viable tumour (30% vs. 0%) was stark, and suggests that the lack of viable tumour seen within post-treatment specimen is directly attributable to DEBIRI-TACE. An indirect effect on the untreated segment cannot be completely ruled out, as the patient received 100mg of irinotecan with the beads. However, pharmacokinetic modelling has suggested systemic exposure after irinotecan eluting beads is low.
and so this seems unlikely. A similar pattern was observed in patient 2, with targeted delivery leading to complete tumour destruction whilst the non-targeted lesion had a large proportion of viable tumour (0% vs. 60%). In both patients 1 and 2, the treated and untreated lesions were of comparable size.

In patient 3, the radiological appearance of a lesion after embolisation suggests an inherent change to the structure of that lesion. Post-resection analysis demonstrated that this lesion had 75% necrosis and 25% fibrosis. It therefore seems likely that the initial effect of irinotecan bead in this lesion was mediated predominantly by occlusion of the blood supply to the tumour rather than the effect of the irinotecan, as chemotherapy-associated change seems to be represented by fibrotic involution rather than necrosis (Poultsides et al., 2012). Interestingly, all the lesions within the treated lobe showed 0% viable tumour. By contrast, the untreated lesion in segment VII demonstrated a large amount of viable tumour (45%) and a very low amount of fibrosis (5%), as would be expected in a chemo-naïve lesion. The large amount of viable tumour observed in lesions supplied by arterial flow proximal to the point of bead release and the absence of tumour in previously unidentified lesions targeted by lobar embolisation support the oncologic rationale for very proximal “whole lobe” embolisation.

Another putative advantage of DEBIRI-TACE is the reduction in off-target effect with reduced systemic toxicity and CALI. Previous data have suggested that exposure to systemic irinotecan is associated with CALI irrespective of BMI (Robinson et al., 2012), with the MD Anderson group identifying steatohepatitis (defined as Kleiner score >4) in 20.2% of patients treated with a median of 16 weeks of FOLFIRI (Vauthey et al., 2006). By contrast, only one patient in this series (2.6%) had evidence of steatohepatitis. As steatohepatitis is independently associated with postoperative mortality (Vauthey et al., 2006), the low reported rates after a single treatment with DEBIRI-TACE are encouraging. A potential role for DEBIRI-TACE is
in combination with systemic FOLFOX for liver-dominant disease in an effort to achieve the impressive response rates reported after the administration of FOLFOXIRI (Falcone et al., 2007). If this is delivered with the intent of bringing irresectable disease to resection, CALI is a significant concern. The low incidence of steatohepatitis after single DEBIRI-TACE suggests this combination may not result in an increase in parenchymal liver damage. However, further work would be required to assess the combined effect of this regimen.

Despite relatively short follow up after resection, median DFS was 379 days with a nominal 1-year DFS of 56%. Analysis of factors associated with worse DFS found synchronous presentation and KRAS status to be predictive on univariate analysis, although only KRAS status was significant on multivariate modelling. Interval between primary tumour and identification of metastases has previously been associated with a negative prognosis and incorporated into risk modelling systems by Fong and Nordlinger (See section 1.10.2).

Importantly, radiological response assessed by both RECIST and morphological response criteria was not associated with disease free survival or with degree of tumour destruction. RECIST remains the current gold standard for assessing the response of solid tumors to anticancer drugs. However, there is growing evidence that RECIST criteria may not optimally reflect tumour response to therapy (Choi et al., 2007), (Chun et al., 2009). Radiological assessment of complete response is also poor, with 80% of lesions demonstrating complete response containing viable tumour (Benoist et al., 2006). Further work to improve radiological assessment of response to DEBIRI is therefore vital. As well as improving morphological and contrast-based criteria, functional imaging (such as PET) may offer better assessment of viable remnant tumour.
The findings of this study need to be viewed with some caution in light of a number of potential confounding factors. The beads used for embolisation varied in size from 100 – 300 µM, with the manufacturer reporting a normal distribution of bead sizes within this range (Taylor et al., 2007). An identical bead slurry should therefore be delivered to every patient. However, differences in the diameter of feeding vessels may result in variations in embolisation distance from the tumour. This in turn may effect the degree of embolic effect, with more distant embolisation less likely to result in total tumour ischaemia. Although there is no in vivo data on drug concentration within tumour after embolisation, it seems plausible that more proximal embolisation would also lead to reduced drug concentration within a larger volume of tumour-containing tissue.

Treatment consisted of a nominal dose of 2ml of bead containing 200 mg of irinotecan. However, actually volume delivered and therefore dose of drug administered varied as the radiological endpoint for delivery was near stasis of contrast. For patients with multiple metastases, this volume of bead slurry may not have been adequate to reach the endpoint for all lesions. This data was not captured by the trial data collection. Embolisation technique also evolved during the study period, from super-selective distal embolisation to lobar administration. Although the maximal volume of drug and radiological endpoint remained the same, it seems likely that the radiological endpoint would be achieved with a lower volume of bead slurry for selective delivery. This change may have led to significant differences in ischaemic effect and local drug concentration between patients.

This single arm study was designed to assess safety of an existing medical product. Although the evidence supporting DEBIRI in the palliative setting is convincing, it remains unclear whether its effectiveness is due to vascular embolisation or the local delivery of chemotherapy. Evidence suggests that fibrotic involution results from chemotherapeutic effect, and necrosis from tissue ischaemia. The significant
degree of tumour necrosis seen within treated lesions suggest that the embolic effect is a significant factor. Direct comparison of bland vs. chemoembolisation with DC bead for CRLM is limited to preclinical animal modeling (Eyol et al., 2008), and tantalising evidence from hepatocellular carcinoma suggests that bland embolisation may be equally effective (Brown et al., 2013). However, these results must be interpreted with caution as hepatocellular carcinoma is a hypervascular disease compared to CRLM. A further randomised study comparing bland DC bead embolisation vs. DEBIRI chemoembolisation would provide a definitive answer to this question.

Although tissue for analysis was sampled using the same protocol for each patient, inherent differences in the surgery patients received may have significantly altered the time from starting the procedure and tissue preservation in liquid nitrogen. A complex resection, involving a significant amount of intraoperative vascular exclusion, would result in the harvested tissue having a prolonged ischaemic time with a possible impact on tumour necrosis. By contrast, a straightforward metastectomy without vascular exclusion would result in much less tissue ischaemia. Operative data captured in the trial protocol did not record precise duration of vascular exclusion, and retrospective review of operative notes routinely reported vascular exclusion time as “less than n minutes”. Assessing the impact of intraoperative devascularisation on tumour response is therefore impossible. One potential approach for future studies would be to perform intraoperative biopsy of tissue and tumour prior to resection i.e. before any devascularisation has taken place. There are concerns about tumour seeding after biopsy, but this could be addressed by ensuring that the biopsy track was excised as part of the eventual resection.

In conclusion, this trial has demonstrated the safety and efficacy of neoadjuvant DEBIRI-TACE is patients with easily resectable colorectal liver metastases. This
treatment can be delivered prior to surgery, with minimal morbidity. The impressive pathological response rates after a single treatment with DEBIRI-TACE are comparable with that seen after multiple cycles of systemic chemotherapy, with no evidence of damage to background hepatic parenchyma. Although follow-up data for this trial is immature and limited by relatively small numbers, KRAS status appears to be prognostic for disease free survival after treatment with DEBIRI-TACE.
3 Development of LC-MS/MS bioassay for the measurement of irinotecan and its key metabolites
3.1 Introduction

Although DEBIRI-TACE has been investigated in preclinical and early clinical studies, the pharmacokinetics and hepatic metabolism of locally delivered irinotecan have not been well characterized. Various methods have been developed for the measurement of irinotecan and its major metabolites in a wide variety of biological matrices (plasma, urine, bile and faeces) from both animal models and humans. Most rely on sample separation by high performance liquid chromatography (HPLC), followed by ultraviolet or fluorescence detection of drug and metabolites (Humerickhouse et al., 2000; de Bruijn et al., 1997; Rivory et al., 1998). There is now growing interest in the coupling of mass spectrometers (MS) to HPLC systems. This technique offers several advantages, including high specificity and unequivocal quantification. To date, HPLC-MS based methods have relied on solid phase extraction techniques to remove sample protein prior to MS analysis (D’Esposito et al., 2008). However, this is both time-consuming and expensive. Although several methods for the LC-MS analysis of irinotecan and one or two metabolites have been reported, only two published LC-MS methods allow for the measurement of irinotecan and its three main metabolites (SN-38, APC and SN-38G) simultaneously. Neither of these methods has been fully validated in human samples (Corona et al., 2010; Chen et al., 2012).

The aim of this study was therefore to develop and validate a novel, specific, rapid and simple LC-MS based assay for the accurate quantification of irinotecan, SN-38, APC and SN-38G in human biological matrices, which avoids the need for solid phase extraction. This validated assay was used to optimise microsomal incubation conditions, allowing further quantitative exploration of the hepatic metabolism of irinotecan.
3.2 Materials

3.2.1 Hardware & reagents

**Applied Biosystems, Carlsbad, CA, USA**

TFA

**Fisher Scientific, Waltham, MA, USA**

Cryodorf vials, H_2O, ACN

**Sigma Aldrich, St. Louis, MO, USA**

Irinotecan, Camptothecin, DMSO, Formic acid, NADPH, UDPGA, MgCl_2, Sodium Acetate, β-glucuronidase, Chromacol autosampler glass vials, KH_2PO_4, Na_2H_2PO_4

**Tocris Bioscience, Minneapolis, MN, USA**

SN-38

**Santa Cruz Biotechnology, Dallas, TX, USA**

APC

**Millipore, Billerica, MA, USA**

Multiscreen Solvinert 96 well filter plate

**Wheaton Glassware, Millville, NJ, USA**

LSL tube
3.2.2 Buffers

Phosphate Buffer

1.78 g KH₂PO₄ and 9.55 g Na₂H₂PO₄ dissolved in 1 litre of ddH₂O, pH titrated to 7 with hydrochloric acid

3.3 Methods

3.3.1 Biosamples

3.3.1.1 Serum sampling

Blood samples for assay validation were taken from a group of 10 control volunteers. Samples were placed on ice immediately after collection, then centrifuged at 640 g for 10 minutes at 4°C. Mixing 10 control sera in equal volume created a pooled reference sample. Serum was placed in a cryodorf vial and snap frozen in liquid nitrogen before storage at -80°C. Samples were thawed at room temperature when required.

3.3.1.2 Hepatic parenchymal sampling

Human microsomal samples for assay validation were generated from a chemo-naive control population of 10 patients undergoing liver resection for hepatic malignancy. Patients received a reverse L laparotomy. To reduce intraoperative blood loss, low venous pressure anaesthesia was used with CVP consistently maintained below 5 cm H₂O. Parenchymal transection was performed using a Cavitron Ultrasonic Surgical Aspirator (CUSA)(Valleylab, Boulder, CO, USA).
Immediately after the specimen had been delivered from the patient, samples of normal hepatic parenchyma were placed in a Cryodorf vial then snap frozen in liquid nitrogen and stored at -80°C. Samples were thawed at room temperature when required.

3.3.2  
**LC-MS/MS assay development**

3.3.2.1  
**Chemicals and reagents**

As purity of the reference standard can affect data quality, an authenticated and high purity reference sample of each analyte was purchased (Table 13).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Source</th>
<th>Externally validated purity</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irinotecan</td>
<td>Sigma Aldrich</td>
<td>&gt;97%</td>
<td>623.1</td>
</tr>
<tr>
<td>SN-38</td>
<td>Tocris Bioscience</td>
<td>&gt;98%</td>
<td>392.4</td>
</tr>
<tr>
<td>APC</td>
<td>Santa Cruz Biotechnology</td>
<td>&gt;99%</td>
<td>618.7</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Sigma Aldrich</td>
<td>&gt;95%</td>
<td>348.4</td>
</tr>
</tbody>
</table>

**Table 13; Source, purity and molecular mass of reference analytes used for assay development.**

SN-38G could not be purchased as a standard, and so an alternative approach to quantification was developed (See section 3.3.4.5). Camptothecin was chosen as internal standard because of structural similarity to the analytes of interest. Formic acid (98%) and DMSO were from Sigma Aldrich. LC-MS grade H₂O and acetonitrile were from Fisher Scientific. Peptide synthesis grade trifluoroacetic acid (TFA) was from Applied Biosystems.

3.3.2.2  
**Preparation of human liver microsomal fractions**

Approximately 1 gram of human hepatic parenchyma was placed in a fluted glass tube stored on ice, and 9 ml of iced phosphate buffer added. The parenchyma was
homogenised using a Kinematic Polytron homogeniser at 3 000 rpm for three 10 second pulses. The homogenate was removed and centrifuged at 14 301 g for 25 minutes at 4°C in a Beckman L-60 Ultracentrifuge. Following centrifugation, the supernatant was transferred to a fresh tube, diluted to 10ml final volume using phosphate buffer and centrifuged for a further 65 minutes at 125 812 g at 4°C. After centrifugation, the supernatant was discarded and the pellet resuspended in a final volume of 10ml of phosphate buffer. A final centrifugation of 65 minutes at 125 812 g was carried out at 4°C and the pellet was transferred into a 2 ml Eppendorf tube and resuspended in 500 µl of phosphate buffer without KCl. Protein concentration was calculated using the method of Lowry (Lowry et al., 1951), and microsomal extract from each of the 10 patients combined in equal proportion to give a final concentration of 50 mg ml-1.

3.3.2.3 Sample preparation

Biological samples (microsomal samples, serum samples) underwent protein precipitation by addition of an equal volume of ice cold 50% acetonitrile:50% methanol (v/v) with 0.005% TFA, containing 40 nM camptothecin as internal standard. The addition of TFA gave a final sample pH of 3, shifting SN-38 into the active lactone form. Samples were vortex mixed for 15 seconds, then centrifuged at 20 130 g at 3°C for 20 minutes. 300µl of supernatant was filtered through a pre-wetted Millipore Multiscreen Solvinert 96-well filter plate. Samples were collected in a clean 96 well plate, after which 100µl was transferred to a sealed 200µl Chromacol glass autosampler vial. 20 µl was injected onto the column.
3.3.2.4 *Preparation of standard calibration curves*

Master stock solutions containing 1 mM of irinotecan, camptothecin and SN-38 were generated. Irinotecan, APC and camptothecin were readily soluble in water with ultrasonification. SN-38 was initially dissolved in dimethylsulfoxide (DMSO) then diluted in water to produce a stock solution with a final concentration of DMSO < 0.03%. Serial dilutions were prepared from these stock solutions to generate calibration and quality control samples. Stock solutions were stored in aliquots at -80°C, and only defrosted when required.

Calibration standards were produced using a blank sample (only matrix), a zero sample (matrix containing only protein and internal standard) followed by 10 samples dissolved in appropriate biological matrix containing a range of final concentrations (5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 150 nM, 200 nM, 300nM, 500 nM). Amount of analyte on column for each concentration is given in table 14.
### Table 14; Amount of analyte on column for each concentration of biosample

<table>
<thead>
<tr>
<th>Sample concentration</th>
<th>Amount of analyte on column</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>0 nmol</td>
</tr>
<tr>
<td>5 nM</td>
<td>50 fmol</td>
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<td>10 nM</td>
<td>100 fmol</td>
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<td>25 nM</td>
<td>250 fmol</td>
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<td>3 pmol</td>
</tr>
<tr>
<td>500 nM</td>
<td>5 pmol</td>
</tr>
</tbody>
</table>

3.3.2.5 *HPLC conditions*

Chromatographic sample separation was performed on a Dionex HPLC stack, using an Ultimate 3000 pump, Ultimate 3000 autosampler and Ultimate 3000 column compartment. An Alltima c18 (150 x 2.1mm, 5µm) column, protected by an Alltima c18 5µm guard column, was used for separation. Chromatographic conditions were optimised by experimentation to achieve good sensitivity and peak symmetry.

Optimal separation was achieved with a dual-buffer technique consisting of an aqueous buffer A (LC-MS grade H₂O with 0.25% formic acid) and an organic buffer B (LC-MS grade acetonitrile with 0.25% formic acid). Formic acid was added as a proton donator and to improve peak resolution. A run time of 15 minutes was used, which included a prolonged rinsing period at the end of the run to eliminate analyte carryover. The buffer gradient (A:B) was 90:10 from 0-1.0 minutes, then switched to
10:90 between 1.0-5.0 minutes, then to 0:100 between 5.0-10.0 minutes, before reversing to 90:10 for 10.0-15.0 minutes (wash phase) (Figure 21).

![Solvent gradient used for HPLC separation of analytes.](image)

**Figure 21** Solvent gradient used for HPLC separation of analytes.

Analytes had nominal elution times of 4.95 minutes (SN-38G), 4.97 minutes (APC), 5.05 minutes (irinotecan), 5.5 minutes (SN-38) and 5.61 minutes (camptothecin) (Figure 22).
Representative chromatogram of 1.5 pmol of on column analyte extracted from microsomal fraction, showing SN-38G eluting at 4.95 minutes (pink peak), irinotecan eluting at 5.03 minutes (blue peak), SN-38 at 5.5 minutes (red peak) and camptothecin at 5.7 minutes (green peak). APC elutes at 4.97 minutes (peak not visible).

3.3.2.6 MS/MS conditions

Analysis was performed on an Applied Biosystems QTRAP 4000 mass spectrometer operating in positive electrospay ionization (ESI) mode, controlled by Applied Biosystems Analyst software version 1.5. Detection parameters were optimized by direct infusion of a 0.1µg ml⁻¹ of analyte in buffer A onto the system with the MS operating in full scan mode. Ion spectra for irinotecan, SN-38, APC and Camptothecin gave protonated molecules ([M+H]+) of m/z 587.4, 393.2, 619.4 and 349.2 respectively (Figure 23). The curtain gas was nitrogen at 35 psi. Ionspray
voltage was 4500 V and the source temperature 550°C. The declustering potential (DP), collision excitation potential (CEP) and collision energy (CX) were optimised for each compound (see table 15). The major precursor/product ion group produced was identified (irinotecan $m/z$ 124.3, SN-38 $m/z$ 349.2, APC $m/z$ 227.2 and camptothecin $m/z$ 305.2). Conditions for the detection of SN-38G were based on the method of Chen et al (Chen et al., 2012). A multiple reaction monitoring (MRM) method using these parameters was developed for further validation.
Figure 23 ESI mass spectra for (A) irinotecan, (B) SN-38, (C) APC and (D) camptothecin showing parent and product ion.
## 3.3.3 LC-MS/MS assay validation

### 3.3.3.1 Calibration method

Method validation was performed in line with US Department of Health & Human Services Food and Drug Administration guidelines, as outlined in Bioanalytical Method Validation; Guidance for Industry (FDA, 2001). Full validation was performed using primary matrix (microsomal extract), with partial validation performed in human serum.

### 3.3.3.2 Selectivity and ion suppression

Selectivity was confirmed by measuring blank samples of different microsomal extracts and serum. Direct interference by endogenous substances was minimal, with no obvious peaks at the elution times of the metabolites of interest (Figure 24). Selectivity was also confirmed at the LLOQ (see section 3.3.3.4). Ion suppression was determined by the comparison of peak areas of pure analytes in mobile phase and peak areas of analyte added to microsomal fractions and blank serum. A small decrease in signal was observed for all analytes, but the decrease was proportionate for analytes and IS and so ion suppression was corrected. Ion suppression was less than 20% for both microsomal and serum samples.

### Table 15 Ion source and analyte dependent MS parameters

<table>
<thead>
<tr>
<th></th>
<th>Irinotecan</th>
<th>SN-38</th>
<th>APC</th>
<th>Camptothecin</th>
<th>SN-38G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor ion (m/z)</td>
<td>587.4</td>
<td>393.2</td>
<td>619.4</td>
<td>349.2</td>
<td>569.1</td>
</tr>
<tr>
<td>Product ion (m/z)</td>
<td>124.3</td>
<td>349.2</td>
<td>227.2</td>
<td>305.2</td>
<td>393.2</td>
</tr>
<tr>
<td>Declustering potential (DP)</td>
<td>131.0</td>
<td>106.0</td>
<td>131.0</td>
<td>91.0</td>
<td>131.0</td>
</tr>
<tr>
<td>Collision excitation potential (CEP)</td>
<td>8.0</td>
<td>19.0</td>
<td>12.0</td>
<td>13.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Collision energy (CX)</td>
<td>53.0</td>
<td>36.0</td>
<td>33.0</td>
<td>33.0</td>
<td>53.0</td>
</tr>
</tbody>
</table>
Figure 24 Selectivity shown by representative chromatograms obtained from (a) blank microsomal fraction and (b) drug-free serum, demonstrating background signal but no discrete peaks that could be falsely identified as analyte.

3.3.3.3 Linearity

Concentration curves were constructed using at a blank sample (matrix sample without internal standard), a zero sample (matrix sample with internal standard) and ten further samples covering the expected metabolite concentration range, including the nominal LLOQ (5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 150 nM, 200 nM, 300nM, 500 nM). The ratio of analyte: internal standard showed a linear relationship throughout the expected range of concentrations (0-500nM) (Figure 25). Concentration curves were only accepted if values were within 15% of actual recordings, or 20% at LLOQ.
Figure 25 Representative standard curves obtained for (a) irinotecan, (b) SN-38 and (c) APC, demonstrating a linear relationship with all calculated values within 20\% of those predicted.
3.3.3.4 Lower limit of quantification

The lower limit of quantification is defined as the concentration at which an analyte can be detected with adequate sensitivity and specificity, an accuracy and precision of >80% and a response at least 5 times greater than background signal. A nominal LLOQ was defined as 5nM (50 fmol on column) for all analytes, and validated in serum and microsomal extract (See table 16, Figure 26 & 27).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detected (\pm SD)</th>
<th>(\text{mean nM})</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal preparation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irinotecan</td>
<td>4.0(\pm 0.2)</td>
<td>80.4%</td>
<td>96.0%</td>
<td></td>
</tr>
<tr>
<td>SN-38</td>
<td>5.5(\pm 0.8)</td>
<td>109.6%</td>
<td>98.6%</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>5.9(\pm 0.4)</td>
<td>119.2%</td>
<td>93.8%</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irinotecan</td>
<td>4.3(\pm 0.1)</td>
<td>85.7%</td>
<td>103.4%</td>
<td></td>
</tr>
<tr>
<td>SN-38</td>
<td>4.7(\pm 0.1)</td>
<td>93.6%</td>
<td>98.4%</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>5.5(\pm 0.7)</td>
<td>109.1%</td>
<td>87.9%</td>
<td></td>
</tr>
</tbody>
</table>

Table 16: Accuracy and precision analysis of human liver microsomes and serum spiked with 4 analytes at LLOQ (5nM). All values met FDA criteria for assay validation.
Figure 26 Representative chromatograph of analytes spiked and then extracted from microsomal matrix at LLOQ, showing peak response at least 5 times greater than background noise. At this concentration, accuracy and precision was >80%.

3.3.3.5 Interbatch accuracy and precision

Accuracy is defined as the closeness of mean test results to expected values, determined by repeated measurements of samples containing known values. Three concentrations were used (LLOQ, mid-range, high-range), with each concentration measured 5 times. Mean value was accepted if it was within 15% of the actual value, except at the LLOQ where 20% was acceptable.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision was measured 5 times at the same 3 different concentrations. Precision met FDA assay
validation standards when values for the coefficient of variation were <15%, or 20% at LLOQ (Table 17 & 18).
<table>
<thead>
<tr>
<th>Actual nM</th>
<th>Irinotecan Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
<th>SN-38 Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
<th>APC Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.0±0.2</td>
<td>80.4%</td>
<td>96.0%</td>
<td>5.5±0.8</td>
<td>109.6%</td>
<td>98.6%</td>
<td>5.9±0.4</td>
<td>119.2%</td>
<td>93.8%</td>
</tr>
<tr>
<td>100</td>
<td>96.3±0.3</td>
<td>96.3%</td>
<td>99.7%</td>
<td>103.7±2.9</td>
<td>103.7%</td>
<td>97.2%</td>
<td>93.9±0.7</td>
<td>93.9%</td>
<td>99.3%</td>
</tr>
<tr>
<td>200</td>
<td>191.4±0.4</td>
<td>95.7%</td>
<td>99.8%</td>
<td>203.8±7.1</td>
<td>101.9%</td>
<td>96.5%</td>
<td>220.4±1.3</td>
<td>110.2%</td>
<td>99.4%</td>
</tr>
</tbody>
</table>

Table 17; Inter-batch accuracy and precision for three microsomal QC samples at LLOQ, mid and high range.

<table>
<thead>
<tr>
<th>Actual nM</th>
<th>Irinotecan Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
<th>SN-38 Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
<th>APC Detected nM (mean±SD)</th>
<th>Acc.</th>
<th>Prec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.3±0.1</td>
<td>85.7%</td>
<td>103.4%</td>
<td>4.7±0.1</td>
<td>93.6%</td>
<td>99.4%</td>
<td>5.5±0.7</td>
<td>109.1%</td>
<td>87.9%</td>
</tr>
<tr>
<td>100</td>
<td>91.4±2.3</td>
<td>91.4%</td>
<td>97.5%</td>
<td>107.4±2.0</td>
<td>107.4%</td>
<td>98.1%</td>
<td>97.6±9.7</td>
<td>97.6%</td>
<td>90.1%</td>
</tr>
<tr>
<td>200</td>
<td>186.4±8.2</td>
<td>93.2%</td>
<td>95.6%</td>
<td>207.0±13.2</td>
<td>103.5%</td>
<td>93.6%</td>
<td>210.6±2.9</td>
<td>105.3%</td>
<td>101.4%</td>
</tr>
</tbody>
</table>

Table 18; Inter-batch accuracy and precision for three serum QC samples at LLOQ, mid and high range.
3.3.3.6 *Intrabatch accuracy and precision*

Once a method has been partially validated, accuracy and precision should be regularly monitored to ensure it continues to operate satisfactorily. Precision and accuracy can therefore be defined as intrabatch (repeat samples within the same run) and interbatch (repeat samples run on different days). Intrabatch accuracy and precision require the same thresholds as interbatch accuracy and precision. These results remained consistent, and within acceptable parameters (Tables 19 and 20).
<table>
<thead>
<tr>
<th>Actual nM</th>
<th>Actual (mean±SD)</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.6±0.2</td>
<td>4.2±0.1</td>
<td>96.3%</td>
<td>83.8%</td>
<td>5.6±3.0</td>
<td>96.9%</td>
<td>96.9%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>113.1±5.5</td>
<td>113.8±2.0</td>
<td>113.8%</td>
<td>111.8%</td>
<td>107.3±3.4</td>
<td>101.8%</td>
<td>107.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>214.2±12.6</td>
<td>114.8±2.8</td>
<td>114.8%</td>
<td>114.8%</td>
<td>202.8±4.3</td>
<td>102.4%</td>
<td>107.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 19; Intra-batch accuracy and precision for 3 microsomal QC samples at LLOQ, mid and high range.

<table>
<thead>
<tr>
<th>Actual nM</th>
<th>Actual (mean±SD)</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Detected nM (mean±SD)</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.2±0.5</td>
<td>5.0±0.4</td>
<td>100.3%</td>
<td>101.8%</td>
<td>5.3±0.3</td>
<td>105.3%</td>
<td>105.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>92.3±8.0</td>
<td>118.7±13.9</td>
<td>118.7%</td>
<td>111.7%</td>
<td>92.5±8.5</td>
<td>92.5%</td>
<td>109.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>196.6±19.1</td>
<td>196.8±12.8</td>
<td>98.4%</td>
<td>106.5%</td>
<td>188.2±0.8</td>
<td>94.1%</td>
<td>99.6%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 20; Intra-batch accuracy and precision for 3 serum QC samples at LLOQ, mid and high range.
3.3.3.7 Analyte recovery

Recovery is defined as the detector response obtained from an amount of the analyte added to and extracted from a biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard in reconstitution solution. Although recovery does not need to be consistently approaching 100%, it is important recovery of both analyte and internal standard is consistent throughout the expected range of results to ensure validity. Recovery experiments were performed by comparing the amount of analyte detected in samples of microsomal extract and serum at three QC concentrations (low, mid and high-range) with unextracted standards dissolved in buffer A that represented presumed 100% recovery. The overall recovery for irinotecan, SN-38 and APC was determined at 3 different QC concentrations (5, 100, 200 nM) (Table 21).

<table>
<thead>
<tr>
<th>nM</th>
<th>Mean irinotecan % recovery</th>
<th>Mean SN-38 % recovery</th>
<th>Mean APC % recovery</th>
<th>Mean camptothecin % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>79.1%</td>
<td>76.9%</td>
<td>78.3%</td>
<td>84.1%</td>
</tr>
<tr>
<td>100</td>
<td>72.8%</td>
<td>76.2%</td>
<td>74.9%</td>
<td>80.1%</td>
</tr>
<tr>
<td>200</td>
<td>73.1%</td>
<td>78.1%</td>
<td>83.2%</td>
<td>78.6%</td>
</tr>
<tr>
<td>5</td>
<td>82.1%</td>
<td>84.2%</td>
<td>83.3%</td>
<td>77.3%</td>
</tr>
<tr>
<td>100</td>
<td>76.3%</td>
<td>77.9%</td>
<td>75.4%</td>
<td>81.2%</td>
</tr>
<tr>
<td>200</td>
<td>84.2%</td>
<td>78.7%</td>
<td>76.4%</td>
<td>88.6%</td>
</tr>
</tbody>
</table>

Table 21 Analyte recovery from microsomal extract & serum

3.3.3.8 Analyte stability

A number of studies have already demonstrated the stability of irinotecan and its metabolites in biological matrices (Sparreboom et al., 1998; de Bruijn et al., 1997). Stability of analytes within matrix was confirmed by sequential sampling of four 100
nM samples after storage at room temperature for 8 hours (the maximum period a sample was likely to remain on the autosampler). Freeze-thaw stability was assessed by subjecting four 100 nM samples to three freeze-thaw cycles (room temperature to -80°C).

Samples re-analysed after 8 hours showed consistent levels of analyte in both protein precipitated serum and microsomal protein matrix. Samples subjected to freeze-thaw cycles showed consistent levels of analyte, indicating that drugs were not degraded by the freeze-thaw cycle (Tables 22 and 23).

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Microsomal protein</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irinotecan nM</td>
<td>SN-38 nM</td>
</tr>
<tr>
<td>0</td>
<td>101.0±2.7</td>
<td>109.2±5.7</td>
</tr>
<tr>
<td>2</td>
<td>103.0±3.1</td>
<td>108.3±8.6</td>
</tr>
<tr>
<td>4</td>
<td>99.6±0.9</td>
<td>109.4±5.9</td>
</tr>
<tr>
<td>6</td>
<td>106.0±1.4</td>
<td>108.1±4.7</td>
</tr>
<tr>
<td>8</td>
<td>112.9±10.4</td>
<td>102.0±3.2</td>
</tr>
</tbody>
</table>

Table 22 Amount of analyte detected in 100 nM standard protein-precipitated matrix stored at room temperature over time (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Irinotecan nM</th>
<th>SN-38 nM</th>
<th>APC nM</th>
<th>Camptothecin nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>110.4±7.3</td>
<td>107.3±2.3</td>
<td>99.4±0.9</td>
<td>112.4±3.8</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>107.3±3.2</td>
<td>99.4±1.5</td>
<td>103.5±1.6</td>
<td>108.6±3.2</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>111.4±6.4</td>
<td>104.7±2.1</td>
<td>107.5±5.1</td>
<td>105.4±1.6</td>
</tr>
</tbody>
</table>

Table 23 Amount of analyte detected in 100 nM standard after three freeze-thaw cycles (mean ± standard deviation)
3.3.4 Microsomal incubation development

Drug was suspended in PO₄ buffer in a 10ml glass LSL tube placed in a 37°C orbital incubator cabinet, and allowed to reach operating temperature over 5 minutes. Reactions were initiated by the addition of warmed microsomal protein to give a total reaction volume of 500 µl. Control samples were run alongside all samples, one containing no protein and one containing no drug. Amount of SN-38 detected in the protein-free sample (control) was subtracted from the amount detected in the incubations. Reactions were terminated after an appropriate time by the addition of 500 ul of 50% ACN; 50% MeoH; 0.005% TFA, containing 40 nM camptothecin.

3.3.4.1 Irinotecan concentration optimisation

Incubations were performed with a microsomal protein concentration of 4 mg ml⁻¹. Incubations were conducted for 30 minutes with agitation at 37°C. Increasing concentrations of irinotecan led to a linear increase in SN-38 production (Figure 28).
Figure 28 Graph showing SN-38 production at increasing concentrations of irinotecan incubated with 4mg ml\(^{-1}\) microsomal protein.

3.3.4.2 Protein concentration optimisation

Incubations were performed with an initial irinotecan concentration of 5 µM. Incubations were conducted for 30 minutes at 37°C. Increasing concentrations of microsomal protein led to a linear increase in SN-38 production (Figure 29). There was a linear relationship between SN-38 production (pmol mg\(^{-1}\) min\(^{-1}\)) and protein concentration (Figure 30), suggesting that at 5µM irinotecan it is the availability of catalytic enzyme that is the rate-limiting step in SN-38 production.
**Figure 29** Graph showing increasing SN-38 production with increasing protein concentration

**Figure 30** Graph showing linear production of SN-38 (pmol mg\(^{-1}\) min\(^{-1}\)) with increasing protein concentration suggesting that protein availability is the rate-limiting step in SN-38 production when incubated with 5 µM irinotecan.
3.3.4.3 Microsomal incubations with co-factors

Incubations were repeated with an initial irinotecan concentration of 5µM, 0.5mg ml⁻¹ microsomal protein with the addition of 1mM NADPH and/or 2mM UDPGA. These cofactors were necessary for the CYP3A4 mediated production of APC and the UGT1A1 mediated production of SN-38G respectively.

A time course was run, with incubations of 0, 10, 20, 30, 60, 120, 180 and 240 minutes. Reactions were terminated by the addition of an equal volume of 50% ACN; 50% MeoH; 0.005% TFA, containing 40 nM of camptothecin (final concentration 20 nM). In all cases, irinotecan consumption was <5%.

SN-38 production was comparable for all incubation conditions, irrespective of co-factors. This shows that at a concentration of 5 µM irinotecan, the interconversion of irinotecan to SN-38 was not affected by the CYP3A4 deactivation of irinotecan and UGT1A1 mediated formation of SN-38G, indicating that the relative capacity of CES pathway far exceeds that of the CYP3A4/UGT1A1 pathway (Figure 31).

Figure 31 Graph showing SN-38 production in the presence of the cofactors UDPGA & NADPH at 5µM irinotecan.
The CYP3A4 mediated conversion of irinotecan to APC was dependent on the presence of NADPH, with no APC produced if this co-factor was not present (Figure 32). The production of APC showed marked plateauing after 60 minutes, presumably because of NADPH exhaustion. The presence of UDPGA, allowing the glucuronidation of SN-38G, did not result in a detectable reduction in APC production, suggesting these pathways are not competitive at this concentration.

Figure 32 Graph showing APC production with the addition of NADPH, a co-factor necessary for the function of CYP3A4. The addition of UDPGA (a co-factor necessary for the UGT1A1 mediated glucuronidation of SN-38) does not appear to lead to competition between these 2 pathways. Plateauing of this curve likely represents exhaustion of NADPH.
SN-38G production only occurred in the presence of UDPGA (Figure 33). Production was linear to 240 minutes, suggesting that the co-factor had not been exhausted by this time point. The presence of NADPH did not reduce the amount of SN-38G produced, suggesting that at a 5µM concentration of irinotecan the CES mediated production of SN-38 was saturated and the CYP3A4 mediated production of APC did not reduce the production of SN-38.

Figure 33 Graph showing the production of SN-38G with the addition of UDPGA, a co-factor necessary for the UGT1A1 mediated glucuronidation of SN-38. NADPH in addition to UDPGA did not lead to competition between these 2 pathways.

3.3.4.4 Physiologically relevant irinotecan concentration optimisation

These experiments were then repeated at a much lower concentration of 500nM irinotecan. 500 nM was used as it was comparable with reported serum irinotecan
concentrations 1 hour after embolisation (See section 4.3.5.1) and thus represented a physiologically relevant concentration.

A similar pattern of analyte production to the 5µM irinotecan incubations was seen, suggesting that at a physiologically relevant dose competition between pathways does not alter the production of the active metabolite SN-38 (Figure 34, 35 & 36).

![Graph showing SN-38 production](image)

**Figure 34** Graph showing SN-38 production irrespective of the addition of enzymatic cofactors with no interpathway competition at a physiologically relevant dose. SN-38 production is approximately 10-fold lower than the 5µM irinotecan incubation.
Figure 35 Graph showing APC production with the addition of NADPH, a co-factor necessary for the function of CYP3A4. The addition of UDPGA does not lead to competition between these 2 pathways at this concentration. APC production is approximately 10-fold lower than for the 5uM irinotecan incubation.
3.3.4.5 Quantification of SN-38G

As SN-38G could not be purchased as an existing standard, an alternative approach to quantification was developed.

A microsomal incubation was performed using 1mg ml⁻¹ protein, 2mM UDPGA, 5mM MgCl₂, and 20µM SN-38. An LC-MS MRM assay was run using M/Z data from previously published data (Chen et al., 2012). This confirmed the detection of a peak that was thought to represent SN-38G. This incubation was repeated for 0, 10, 20, 30, 60, 120 and 180 minutes, both with and without UDPGA.

The incubation containing UDPGA clearly demonstrated a linear increase in signal intensity for presumed SN-38G. This signal was absent when UDPGA was not added (Figure 37).
Figure 37 Graph showing increasing SN-38G signal as SN-38 is incubated with hepatic microsome and UDPGA. This signal is absent for incubations without UDPGA.

3.3.4.6 β-glucuronidase mediated production of SN-38

This incubation was then repeated, with the sample allowed to incubate for 660 minutes to maximise conversion of SN-38 to SN-38G. Confirmation that this signal was SN-38G was achieved by the addition of 40 000 Fishmann units of β-glucuronidase dissolved in 0.1M sodium acetate buffer. This incubation was then conducted for a further 440 minutes, and the loss of signal recorded (Figure 38). The reducing signal intensity corresponded to the back conversion of SN-38G to SN-38.
Figure 38 Graph showing the formation of SN-38G in human liver microsome incubated with UDPGA. The addition of β-glucuronidase results in a rapid loss of signal as SN-38G is back-converted to SN-38.

In order to quantify the amount of SN-38G, a stoichometric method was used. A series of SN-38 standards were made, with concentrations ranging from 0 – 200 nM. These standards were then incubated with 1mg ml⁻¹ protein, 2mM UDPGA and 5mM MgCl₂ for 5 hours. There was a linear increase in SN-38G formation with increasing SN-38 concentration (Figure 39).
Figure 39 Graph showing SN-38G formation (AUC) increases with increasing concentrations of SN-38.

The same QC standards were then analysed without protein or UDPGA. The difference in SN-38 measured between the incubated and non-incubated sample ([SN-38]\textsubscript{QC} - [SN-38]\textsubscript{incubation}) was assumed to be the amount of SN-38G produced, and correlated with signal intensity. This standard curve was then used for all future estimations of SN-38G concentration (Figure 40).
Figure 40 Standard curve used for the quantification of SN-38G in future incubations. Using a stoichiometric approach, the difference in SN-38 detected in the incubated and non-incubated samples was assumed to represent the amount of SN-38G produced. This was then plotted against signal intensity, and this relationship used to quantify all future measurements of SN-38G production.

3.3.4.7 Summary of optimal assay conditions for analysis of human serum

200µl of 50% ACN; 50% MeoH; 0.005% TFA containing 40 nM camptothecin as internal standard was added to 200 µl of serum to precipitate the plasma protein fraction. pH of the stop buffer was <3, converting all drug and metabolites into the active lactone form. Samples were vortex mixed for 15 seconds, then centrifuged at 20 130 g at 3°C for 20 minutes. 300µl of supernatant was filtered through a pre-wetted Millipore Multiscreen Solvinert 96 well filter plate and collected in a clean 96 well plate. 100µL of the sample was transferred to a sealed 200µl Chromacol glass autosampler vial and 20 µL injected onto the column. Amount of drug and analyte detected was normalised to the amount of internal standard. All measurements were performed 3 times.
3.3.4.8 Summary of optimal microsomal drug incubation conditions

Incubations consisted of 500nM irinotecan, 2mM UDPGA and 1mM NADPH in 500 µl of PO₄ buffer. Incubation mixtures were pre-warmed in a 10ml glass SDS tube suspended in a 37°C agitating water bath, and allowed to reach operating temperature over 5 minutes. Reactions were initiated by the addition of warmed microsomal protein (final concentration 0.5mg ml⁻¹). Reactions were terminated after 120 minutes by the addition of 500µl of 50% ACN; 50% MeOH; 0.005% TFA containing 40 nM camptothecin as internal standard. Control samples were run alongside all samples, one containing no protein and one containing no drug. Amount of analyte detected in the protein-free sample (control) was subtracted from the amount detected in the incubations. Samples were vortex mixed for 15 seconds, then centrifuged at 20 130 g at 3°C for 20 minutes. Samples were filtered as described above and injected onto the column. Amount of drug and analyte detected was normalised to the amount of internal standard. All reactions were completed three times.
3.4 Discussion

This study shows the development of a novel one-step protein precipitation-extraction LC-MS/MS assay for the quantitative detection of irinotecan, SN-38, APC and SN-38G in a variety of human biological matrices. The assay was fully validated to FDA standards. The assay is sensitive, specific, accurate, and reproducible, and was successfully used for the optimization of microsomal incubation conditions.
4 Metabolic profiling to identify predictive biomarkers for DEBIRI
4.1 Introduction

Transcatheter embolic therapy with irinotecan-eluting beads (DEBIRI) allows the targeted delivery of irinotecan to patients with CRLM. PARAGON Bead is a next generation investigational product where the manufacturer preloads the embolic bead with a standardized dose of irinotecan. Irinotecan is a water-soluble prodrug converted into the active agent SN-38 by carboxylesterases 1 & 2 (CES-1 & CES-2 (Guichard et al., 1999)). SN-38 directly targets the DNA repair enzyme topoisomerase I (Topo-1) promoting apoptotic cell death (Hsiang et al., 1985) leading to tumour replacement with fibrosis. Patients who have a large fibrotic response to chemotherapy demonstrate better overall survival (Rubbia-Brandt et al., 2007) (Blazer et al., 2008) (Poultsides et al., 2012) and so it is vital that patients are treated with the most effective chemotherapeutic regimen possible. Irinotecan and SN-38 both undergo metabolic inactivation; irinotecan is deactivated by CYP3A4 to the metabolite APC, whilst SN-38 is conjugated to glucuronic acid by UDP-glucuronyl transferase 1A1 (UGT1A1) (Figure 4.1). A complex balance between the activities of multiple hepatic enzymes therefore determines the overall exposure to SN-38. There is wide interpatient variation in expression of these key enzymes. Levels of CES-2 show 15-fold variation in hepatic parenchyma (Xu et al., 2002) and 23-fold within colorectal cancer (Sanghani et al., 2003), levels of CYP3A4 can be affected by environmental exposure (Lau et al., 2011) and the promoter of UGT1A1 is subject to a common and functionally relevant polymorphism (Iyer et al., 2002). Perhaps unsurprisingly there is large interpatient variability in the pharmacokinetics and metabolism of irinotecan (Mathijssen et al., 2004).

This study assessed serum concentrations of irinotecan and its metabolites after administration of DEBIRI, as well as investigating the hepatic metabolism of
irinotecan. These metabolic factors were then correlated with response to treatment to identify potential clinically relevant predictive factors.

Figure 41 Diagram summarizing the metabolism of irinotecan and co-factors necessary for metabolite production. Irinotecan is converted into the active metabolite SN-38 by carboxylesterase (CES-1 and CES-2). No co-factors are necessary for this step. Irinotecan is converted to its inactive metabolite APC by the enzyme CYP3A4. This step requires the cofactor NADPH. SN-38 is deactivated by glucoronidation mediated by UGT1A1, which requires the cofactor UDPGA.
4.2 Methods

4.2.1 Patients

This study was the translational arm of the PARAGON II trial, which assessed the feasibility and safety of a single treatment with neoadjuvant DEBIRI before resection of CRLM with an endpoint of pathological tumour response (See section 2.2.5). All patients had resectable CRLM and had not previously been exposed to irinotecan. Tumours were embolised with a nominal dose of 200mg irinotecan using PARAGON Bead (Biocompatibles UK Limited) as previously described (See section 2.3.2).

4.2.2 Biosampling

4.2.2.1 Peri-TACE serum sampling

Blood samples were taken from patients treated with DEBIRI-TACE immediately pre-treatment, one and six hours after embolisation and again at the time of surgery. Samples were immediately placed on ice until centrifugation at 2000 g for 10 minutes at 4°C. Serum was removed, placed in a Cryodorf vial and snap frozen in liquid nitrogen before storage at -80°C until analysis. Time from sampling to snap freezing was consistently less than 30 minutes.

4.2.2.2 Hepatic parenchymal sampling

Immediately after the resected specimen had been removed from the patient, samples of normal hepatic parenchyma and colorectal liver metastasis were placed
in a Cryodorf vial and snap frozen in liquid nitrogen and stored at -80°C. Time from sample removal to snap freezing was consistently less than 5 minutes.

4.2.3 Pathological response scoring

Haematoxylin and eosin stained tumour specimens were reviewed by an experienced hepatobiliary pathologist and assessed according to UK Royal College of Pathologists Liver Resection Standard Dataset (Wyatt et al., 2012) (See section 2.2.5). The pathologist was blinded to patient outcome. Tumour response was graded using a validated system (Blazer et al., 2008). Briefly, the amount of residual cancer was assessed semi quantitatively by estimating the proportion of residual cancer cells, necrotic tissue and fibrosis in relation to the total area of cancer (Figure 42). The location of the beads within tumour was not assessed.

Figure 42 Representative photomicrographs of colorectal liver metastases treated with DEBIRI showing differing pathological responses (A) 30% tumour, 60% fibrosis, 10% necrosis and (B) 0% tumour, 60% fibrosis, 40% necrosis. The beads are clearly visible in the vasculature (black arrows), with areas of necrosis (star), fibrosis (arrow head) and viable tumour (white arrow). Magnification x 4.
4.2.4 Instrumentation, chromatographic conditions and assay validation

An LC-MS/MS assay for the measurement of irinotecan and its metabolites was established and fully validated to FDA standards (FDA, 2001) (See section 3). Samples were separated using an Ultimate 3000 HPLC stack (Dionex) with an Altima C18 (150 x 2.1mm, 5µm) column (Grace). Mass spectrometric detection was performed on a 4000 QTRAP system (ABSciex) operating in a positive electrospray ionization (ESI) mode.

4.2.5 Serum drug concentrations

Serum samples underwent protein precipitation as previously described (See section 3.3.4.7). Analyte concentrations were calculated by linear regression using the peak area ratios of the analyte/internal standard. All measurements were performed in triplicate.

4.2.6 Microsomal preparations & drug incubation conditions

Microsomal incubations were performed as previously described (See section 3.3.4.8). In brief, incubation mixtures (500nM irinotecan, 2mM UDPGA and 1mM NADPH in 500 µl of phosphate buffer, pH 7) were pre-warmed for 5 minutes then initiated by the addition of warm microsomal protein (final concentration 0.5mg ml⁻¹) and terminated after 120 minutes by the addition of 500µl of extraction buffer. Metabolite activity was linear under these conditions (See section 3.3.4). Control samples were run alongside all samples, one containing no protein and one containing no drug. All incubations were run in triplicate.
4.2.7 Targeted and global proteomic quantification

4.2.7.1 Global proteomic analysis using iTRAQ (Isobaric Tag for Relative and Absolute Quantification)

Snap frozen hepatic parenchyma was thawed then mechanically homogenised in dissolution buffer (0.5M TEAB (tetraethylammonium bromide)/0.1% SDS in H2O). Following centrifugation at 2000 g for 20 minutes at 4°C, the supernatant was removed and protein concentration determined by sequential Lowry assay (Lowry et al., 1951).

100 µg of protein was added to 20 µl of dissolution buffer (Applied Biosystems) and reduced with tris(2-carboxyethyl)phosphine at 60°C for 1 hour. Cysteine sulfhydryls were then blocked by the addition of methyl-methanethiosulfonate at room temperature for 10 minutes. Proteins were digested by incubating with 10µl trypsin (Promega Trypsin Gold reconstituted in MilliQ water as per manufacturers instructions) overnight at 37°C.

iTRAQ reagents (AB Sciex) were reconstituted in isopropanol and added to the appropriate sample tube. Following incubation for 2 h at room temperature, protein digests were combined and pH adjusted to <3 by addition of concentrated phosphoric acid and immediately underwent strong cation exchange, followed by LC-MS/MS using an AB Sciex tripleTOF 5600 mass spectrometer (Applied Biosystems). The accumulated LC-MS/MS data was analysed using Protein Pilot (Applied Biosystems). A common reference pool generated from 10 matched human liver samples was used to allow comparison of relative quantification between iTRAQ channels.
4.2.7.2 Targeted proteomic analysis using Western immunoblotting

4.2.7.2.1 SDS-PAGE gel production

Microsomal samples were prepared (See section 3.3.2.2), mixed with 2 x Lammelli buffer and boiled at 100°C for 10 minutes. 12% SDS-PAGE gels were generated using standard laboratory protocol, and 40µg of protein in a final volume of 15µl added to each channel. A further channel containing a common reference pool generated from 10 matched microsomal samples was run on each gel to allow intergel comparison. Seeblue molecular weight markers (Sigma) were run to aid analysis of the molecular weight of protein samples. Gels were subjected to electrophoresis at 150 volts and unlimited ampage until the blue dye front was at the bottom of the gel.

4.2.7.2.2 Western immunoblotting

After electrophoresis, gels were washed in transfer buffer (1 x running buffer in 20% methanol) and proteins transferred to a Hybond nitrocellulose sheet (GE Lifescience) using a blotting sandwich in a transfer unit containing transfer buffer. After transfer, the nitrocellulose membrane was gently washed in water for 5 minutes then transfer of proteins assessed using Ponceau S stain. As microsomal extracts have low levels of suitable loading proteins (e.g. actin), comparable gel loading for all Westerns was verified by visual inspection of the Ponceau stain.

The membrane was rinsed in TST (0.01M Tris-HCL, pH8, 0.15M NaCl, 1% TWEEN 20) to remove the stain then incubated for 1 hour at room temperature with constant agitation in 5% (w/v) milk solution consisting of Marvel milk powder in 1x TBST (20mM Tris-Cl pH 7.6, 150mM NaCl, 0.1% Tween 20) to block non-specific antibody binding sites.
4.2.7.2.3 Antibody incubation conditions

Primary antibody conditions and concentrations were optimized by experimentation. Primary antibody against the protein of interest was added to fresh 1% milk solution at the appropriate dilution (table 24) and incubated with the membrane overnight at 4°C under constant agitation. The membrane was washed 3 times for 5 minutes in 1 x TBST, and then incubated in fresh 1% milk solution with the appropriate secondary antibody (IRDye 680 LT (Li-Cor) 1:20 000, apart from UGT1A1 where 1:5 000 used) for 1 hour at room temperature, with constant agitation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reference</th>
<th>Manufacturer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CES-1</td>
<td>Anti CES-1</td>
<td>Sigma-Aldrich</td>
<td>1:5 000</td>
</tr>
<tr>
<td>CES-2</td>
<td>Anti CES-2</td>
<td>Sigma-Aldrich</td>
<td>1:10 000</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Anti-UGT1A1</td>
<td>AbCam</td>
<td>1:1 000</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Anti-human CYP3A4</td>
<td>BD Biosciences</td>
<td>1:3 000</td>
</tr>
</tbody>
</table>

Table 24 Primary antibodies used for Western blotting. Antibody concentration and incubation times were optimized by experimentation.

4.2.7.2.4 Western blot visualisation and quantification

Blots were visualized and quantified using the LI-COR Odyssey CLx system (LI-COR Biosciences). For each channel, a ratio of sample:reference pool was calculated. All blots were performed at least 4 times, and a mean sample:reference value calculated.
4.2.8 UGT1A1 genotyping

4.2.8.1 DNA extraction

Genomic DNA was extracted from fresh frozen liver using a salt precipitation protocol (Gustincich et al., 1991). Approximately 10 mg of fresh frozen hepatic parenchyma was finely chopped and placed in an Eppendorf tube with an equal volume of Trizol (Invitrogen). Tissue was mechanically homogenized using a Qiagen tissue ruptor. 200 µl of chloroform (Sigma) was added to the sample followed by centrifugation at 2000 g for 15 minutes at 4°C. The clear supernatant containing the DNA fraction was pipetted off and mixed with an equal volume of ethanol to precipitate out DNA. This mix was centrifuged again to create a DNA pellet that was washed twice in 0.1M sodium citrate/10% ethanol. After air-drying, the DNA pellet was resuspended in 50µl of Tris EDTA. DNA quality was quantified using a Nanodrop 8000 spectrophotometer (Thermoscientific). By measuring the relative UV absorbance at wavelengths of 260nm (DNA) and 280nm (protein) it was possible to assess the DNA purity of each sample. A 260:280 ratio >1.8 was considered sufficient for further analysis. Samples were air-dried then re-suspended in ddH₂O to a final concentration of 20ng ml⁻¹.

4.2.8.2 Primer development

Primers (Life Technologies) were designed using Primer BLAST (available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and underwent BLAST quality control prior to use (Forward-5'-CTTGGTGATCGATTGTTTTGC-3', Reverse-5'-AGAGGTTCGCCCTCTCTCTAC-3'). PCR samples were prepared (see table 25) to give a final primer concentration of 0.5 µM.
<table>
<thead>
<tr>
<th>Units</th>
<th>10 x PCR Buffer</th>
<th>5 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25mM MgCl$_2$</td>
<td>2 µL</td>
</tr>
<tr>
<td></td>
<td>dNTP Mix (25mM)</td>
<td>0.4 µL</td>
</tr>
<tr>
<td></td>
<td>Primer Forward (10µM)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td></td>
<td>Primer Reverse (10µM)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td></td>
<td>dd H$_2$O</td>
<td>35.35 µL</td>
</tr>
<tr>
<td></td>
<td>Sample DNA (20ng µl$^{-1}$)</td>
<td>2 µL</td>
</tr>
<tr>
<td></td>
<td>Final Volume</td>
<td>49.75 µL</td>
</tr>
</tbody>
</table>

Table 25 Content of PCR incubation samples. The addition of 1.5 units of Taqman HotstartDNA polymerase (in a volume of 0.25 µL) gave a final incubation volume of 50 µL.

4.2.8.3  PCR conditions

The final reaction conditions included 10 µM forward and reverse primer and 1.5 units of Taqman HotstartDNA polymerase (in a volume of 0.25 µL) (Applied Biosystems) in a final incubation volume of 50 µl. Thermocycling was performed on a GSTORM GS4822 PCR thermocycler (Labtech) using the following conditions: 5 minutes denaturation at 95°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, and a final 7 minute extension step at 72°C.

4.2.8.4  Analysis of PCR products

PCR products were separated on a 3% agarose gel (NuSieve) with ethidium bromide staining. Invitrogen Plus DNA control ladder was used to aid identification of PCR products. Blank PCR incubation mix (without sample DNA) provided the control. The gel was resolved at 120 v for ~ 2.5 hours and visualized on an
ultraviolet light box. PCR products were further quantified using an Agilent 2100 Bioanalyser. Each sample (1µl) was loaded onto an Agilent 16 well DNA chip, followed by 5µl of high sensitivity DNA marker to each sample channel. Blank PCR incubation mix was used as a control channel on each chip. All samples were analysed in duplicate.

4.2.9 Statistical analysis

Statistical analysis was performed using SPSS Statistics version 20 (IBM). All tests were bidirectional. Linear correlation between continuous variables was compared using Pearson coefficient of correlation. Mann-Whitney U-test was used to assess the relationship between continuous pharmacological variables (serum concentrations) and patient groups defined by median protein expression. p<0.05 was considered to represent statistical significance.
4.3 Results

4.3.1 Biosampling

Complete sets of blood samples were obtained from eight patients who underwent neoadjuvant DEBIRI. Both tumour and normal parenchymal tissue were harvested from all eight patients at the time of surgery. In addition, another 2 patients provided tumour and parenchymal samples but had already received DEBIRI at the time recruitment started and so these did no undergo serum sampling (Table 26).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose of DEBIRI-TACE (mg)</th>
<th>Serum sampling</th>
<th>Tissue sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-TACE</td>
<td>1 hr</td>
</tr>
<tr>
<td>Patient 1</td>
<td>80</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Patient 2</td>
<td>64</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 3</td>
<td>80</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 4</td>
<td>150</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 5</td>
<td>160</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 6</td>
<td>90</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Patient 7</td>
<td>175</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 8</td>
<td>105</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 9</td>
<td>130</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Patient 10</td>
<td>100</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 26 Biosampling record sheet for patients recruited into translational arm of PARAGON II study
4.3.2 Pathological response rates

Pathological response was evaluated in all ten patients. Median residual tumour was 30% (range 10-50%), median necrosis 40% (range 40-60%) and median fibrosis 20% (range 10-40%) (Table 27).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumour viability (%)</th>
<th>Tumour necrosis (%)</th>
<th>Tumour fibrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>40%</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>Patient 2</td>
<td>30%</td>
<td>30%</td>
<td>40%</td>
</tr>
<tr>
<td>Patient 3</td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
</tr>
<tr>
<td>Patient 4</td>
<td>10%</td>
<td>50%</td>
<td>40%</td>
</tr>
<tr>
<td>Patient 5</td>
<td>20%</td>
<td>40%</td>
<td>40%</td>
</tr>
<tr>
<td>Patient 6</td>
<td>40%</td>
<td>20%</td>
<td>40%</td>
</tr>
<tr>
<td>Patient 7</td>
<td>40%</td>
<td>40%</td>
<td>20%</td>
</tr>
<tr>
<td>Patient 8</td>
<td>30%</td>
<td>50%</td>
<td>20%</td>
</tr>
<tr>
<td>Patient 9</td>
<td>30%</td>
<td>60%</td>
<td>10%</td>
</tr>
<tr>
<td>Patient 10</td>
<td>50%</td>
<td>40%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Table 27 Tumour pathological response rate after neoadjuvant treatment with DEBIRI-TACE.

4.3.3 Targeted and global proteomic analysis

4.3.3.1 iTRAQ quantification

iTRAQ analysis of whole hepatic parenchyma demonstrated a wide variation in protein expression, with \( \approx 3 \)-fold change in CES-2, \( \approx 7 \)-fold change in CES-1, a \( \approx 5 \)-fold change in CYP3A4 and \( \approx 3 \)-fold change in UGT1A1 (Table 28).
<table>
<thead>
<tr>
<th></th>
<th>CES-2 expression (AU)</th>
<th>CES-1 expression (AU)</th>
<th>CYP3A4 expression (AU)</th>
<th>UGT1A1 expression (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>1.12</td>
<td>2.49</td>
<td>1.20</td>
<td>1.27</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1.42</td>
<td>2.61</td>
<td>1.19</td>
<td>1.84</td>
</tr>
<tr>
<td>Patient 3</td>
<td>1.56</td>
<td>1.89</td>
<td>3.28</td>
<td>2.31</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1.38</td>
<td>1.61</td>
<td>1.61</td>
<td>1.84</td>
</tr>
<tr>
<td>Patient 5</td>
<td>1.42</td>
<td>2.11</td>
<td>1.37</td>
<td>1.42</td>
</tr>
<tr>
<td>Patient 6</td>
<td>2.83</td>
<td>0.41</td>
<td>0.75</td>
<td>1.05</td>
</tr>
<tr>
<td>Patient 7</td>
<td>1.09</td>
<td>1.77</td>
<td>1.91</td>
<td>0.85</td>
</tr>
<tr>
<td>Patient 8</td>
<td>1.84</td>
<td>1.42</td>
<td>2.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Patient 9</td>
<td>1.28</td>
<td>2.97</td>
<td>2.15</td>
<td>1.32</td>
</tr>
<tr>
<td>Patient 10</td>
<td>0.90</td>
<td>3.16</td>
<td>1.13</td>
<td>1.84</td>
</tr>
</tbody>
</table>

Table 28 Parenchymal protein expression by iTRAQ. Numbers are relative expression compared to a common reference channel containing a pool of 10 control human hepatic microsomes.

#=UGT1A1*28 6/7 heterozygotes

4.3.3.2 Western blotting

Western blotting of microsomal protein demonstrated a single band at the expected molecular weight for each target protein (See Figure 43). Wide variation in protein expression between patients was observed. CES-2 expression varied ≈ 6.7 fold, with much lower variation in expression of CES-1 (≈2 fold), UGT1A1 (≈1.9 fold) and CYP3A4 (≈1.7 fold) (Table 29).
Figure 43 Representative Western blots (A) and column charts showing densitometry data of 10 microsomal samples blotted for (B) CES-1, (C) CES-2, (D) CYP3A4 and (E) UGT1A1. Antibody conditions were optimized by experimentation. A combined reference pool of 10 control human hepatic microsomes was run on each gel to allow inter-gel comparison. Densitometry shows mean value from 4 blots (Bar = SD).
Table 29 Microsomal protein expression (±SD) by Western immunoblotting (n=4 different blots). The value is densitometry result compared to common reference pool.

<table>
<thead>
<tr>
<th></th>
<th>CES-2 densitometry (AU)</th>
<th>CES-1 densitometry (AU)</th>
<th>CYP3A4 densitometry (AU)</th>
<th>UGT1A1 densitometry (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.92±0.20</td>
<td>1.34±0.08</td>
<td>1.07±0.17</td>
<td>0.89±0.07</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1.48±0.33</td>
<td>1.05±0.06</td>
<td>1.23±0.23</td>
<td>1.14±0.54</td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.49±0.12</td>
<td>0.74±0.10</td>
<td>1.01±0.09</td>
<td>0.88±0.03</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1.18±0.24</td>
<td>0.83±0.11</td>
<td>0.92±0.23</td>
<td>0.95±0.04</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.79±0.18</td>
<td>0.93±0.10</td>
<td>0.75±0.12</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>Patient 6</td>
<td>2.93±0.82</td>
<td>0.68±0.15</td>
<td>0.80±0.06</td>
<td>0.77±0.06</td>
</tr>
<tr>
<td>Patient 7</td>
<td>0.76±0.11</td>
<td>0.75±0.11</td>
<td>0.96±0.14</td>
<td>0.82±0.12</td>
</tr>
<tr>
<td>Patient 8</td>
<td>0.91±0.10</td>
<td>0.77±0.09</td>
<td>0.87±0.13</td>
<td>0.78±0.05</td>
</tr>
<tr>
<td>Patient 9</td>
<td>0.81±0.10</td>
<td>1.01±0.05</td>
<td>1.18±0.29</td>
<td>1.00±0.33</td>
</tr>
<tr>
<td>Patient 10</td>
<td>0.44±0.09</td>
<td>1.21±0.14</td>
<td>0.71±0.05</td>
<td>1.48±0.78</td>
</tr>
</tbody>
</table>

# UGT1A1*28 6/7 heterozygotes

4.3.3.3 Comparison of protein quantification between iTRAQ and Western immunoblotting

Differences in protein expression between samples using both iTRAQ and Western immunoblotting were concordant for CES-1 ($r^2=0.62$, p=0.007) and CES-2 ($r^2=0.75$, p=0.002). For example patient 6 showed the highest CES-2 expression and lowest values for CES-1 by iTRAQ analysis, which was confirmed by Western blotting. Concordance was less marked for CYP3A4 ($r^2=0.08$, p=0.48) or UGT1A1 ($r^2=0.25$, p=0.146) (Figure 44) and may reflect lack of absolute specificity of the isoform specific antibody used for Western blotting. However, the high concordance in protein quantification between 2 discrete techniques clearly validates the quantification of CES-1 and CES-2 in protein samples.
Figure 44 Scatter plot showing comparison of protein quantification using Western immunoblotting and iTRAQ. CES-1 and CES-2 demonstrated a highly statistically significant relationship between detection by WB and iTRAQ (p=0.007 and 0.002 respectively). In contrast, expression of CYP3A4 and UGT1A1 by Western blot and iTRAQ did not correlate (p=0.48 and p=0.15 respectively).
4.3.4 UGT1A1 genotyping

4.3.4.1 Agarose gel visualisation

Wild type UGT1A1*28 (6/6) heterozygotes were predicted to give a PCR product 62 base pairs long. In the case of a TATA repeat, a wider band would be anticipated on the agarose gel as a *28 (6/7) (heterozygous) or *28 (7/7) (homozygous) would give a product 2 or 4 base pairs larger than the wild type *28 (6/6). Initial visual inspection suggested samples 3, 7 and 9 produced a broader product band than the other samples (Figure 45).

![Agarose gel stained with ethyl bromide to visualize products of UGT1A1*28 PCR. The control channel shows no PCR product. All patient samples show a product of approximately 60 base pairs in size. Lanes 3, 7 and 9 show a broader product band, suggesting possible 6/7 or 7/7 repeat.](image)

4.3.4.2 Agilent 1200 bioanalysis

Further quantitative analysis was performed on an Agilent 2100 Bioanalyser. Visual inspection of the agarose gel suggested patients 3, 7 and 9 had a broader product band. These samples were therefore loaded in duplicate onto an Agilent DNA Bioanalysis chip.
Microcapillary gel analysis clearly demonstrated a broader band for patients 3, 7 and 9. This was confirmed on the in-chip repeats, and the same results were seen on the second chip (Figure 46).

Figure 46 Microcapillary gel from Agilent Bioanalyser 2100 of showing products of UGT1A1*28 PCR. The gel shows a wider product band for patient samples 3, 7 and 9 suggesting a larger PCR product as would be expected in the presence of an SNP. These samples were repeated within the same DNA chip, and show a similar pattern.

Electropherograms from the DNA chip showed a clear spike on the peak representing the PCR product in samples 3, 7 and 9. This spike was seen in the duplicate runs, as well as the second chip, but was not present in other samples. This spike represents the larger PCR product produced by the *28 (6/7) (heterozygous) TATA repeat SNP (Figure 47).
Seven patients were therefore wildtype (6/6) (70% of population), with three patients heterozygotes (7/6) (30% of population).

Figure 47 Representative electropherograms for UGT1A1*28 PCR products. (A) patient sample 1, (B) patient sample 3, (C) patient 7 and (D) patient 9. The left hand peak in each window is the lower DNA marker. The large peak is the 62 base pair PCR product. The clear upward deflection on the large peak seen in samples 3, 7 and 9 represents the 64 base pair product produced by a TA repeat (UGT1A1*28 (6/7). This upward deflection was not seen in other samples (UGT1A1*28 (6/6).

4.3.4.3 Effect of UGT1A1*28 SNP on protein expression

The three patients found to be heterozygous carriers of the UGT1A1*28 6/7 mutation are denoted in table 28 & 29 with a # symbol. There was no difference in level of UGT1A1 expression between patients who were 6/6 and 6/7 using Western blotting (p=0.83) or iTRAQ (p=0.76).

4.3.5 Serum metabolic profiling

4.3.5.1 Serum concentrations of drug and metabolites

Serum drug concentrations varied widely (See table 30, Figure 48), with a median irinotecan concentration of 188.3 nM (range 80.2 - 352.7) at 1 hour and 83.3 nM (38.5-
207.7) at 6 hours. SN-38 concentrations were much lower than irinotecan, with a median of 11.2 nM (6.9-31.2) at 1 hour and 6.2 nM (3.9-10.6) at 6 hours. SN-38G was detected at higher levels than SN-38, with 55.1 nM (1.6-168.6) at 1 hour and 21.2 (1.1-57.5) at 6 hours. APC reached a median concentration of 84.5 nM (12.8-237.0) at 1 hour, with 31.7 nM (12.5-178.9) at 6 hours. No drug or metabolites were detected in serum taken at the time of resection. Individual patient serum concentrations varied widely.
<table>
<thead>
<tr>
<th>Patient</th>
<th>1 hour</th>
<th>6 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Patient 2 ♯</td>
<td>80.2</td>
<td>30.2</td>
</tr>
<tr>
<td>Patient 3 ♯</td>
<td>105.1</td>
<td>50.9</td>
</tr>
<tr>
<td>Patient 4</td>
<td>277.5</td>
<td>88.0</td>
</tr>
<tr>
<td>Patient 5</td>
<td>341.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Patient 6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Patient 7 ♯</td>
<td>352.7</td>
<td>207.7</td>
</tr>
<tr>
<td>Patient 8</td>
<td>187.3</td>
<td>80.9</td>
</tr>
<tr>
<td>Patient 9</td>
<td>189.3</td>
<td>166.2</td>
</tr>
<tr>
<td>Patient 10</td>
<td>80.0</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Table 30 Mean serum drug concentrations 1- and 6-hours post-DEBIRI (n=3 separate measurements). NA=patients were not sampled for serum as they had already received DEBIRI and were awaiting resection when recruitment started

♯=UGT1A1*28 6/7 heterozygotes
Figure 48 Composite graphs showing serum concentrations of (A) irinotecan, (B) SN-38, (C) SN-38G and (D) APC for 8 patients at 0, 1 and 6 hours post-DEBIRI. No drug or metabolite was detectable in any patients at 30 days.
There was a clear correlation between dose of DEBIRI and 1 and 6-hour serum irinotecan concentrations ($r^2=0.89$ $p=0.0001$ and $r^2=0.65$ $p=0.016$ respectively, see Figure 49). There was no correlation between dose of administered DEBIRI and SN-38 at 1 and 6 hours ($r^2=0.15$, $p=0.72$ and $r^2=0.36$, $p=0.39$)

![Figure 49 Scatter plot showing correlation between dose of DEBIRI and serum concentrations of irinotecan at 1 and 6 hours.](image)

To correct for variations in delivered drug dose, ratios of SN-38 to irinotecan were calculated for each patient. In all eight patients the ratio of metabolites was comparable at 1 and 6 hours (table 30, Figure 48). However, there was wide interpatient variation in these ratios, suggesting differences in the activation of irinotecan between patients.

There was no clear relationship between serum concentration of irinotecan and SN-38 ($r^2=<0.01$, $p=0.92$) or APC ($r^2=0.07$, $p=0.29$), or SN-38 and SN-38G ($r^2=0.27$, $p=0.51$). There was no difference in the ratio of SN-38G:SN-38 between UGT1A1*28 6/6 and 6/7 patients ($p=0.79$).
Figure 50 Column chart showing ratio of SN-38:irinotecan measured in serum 1 and 6 hours after TACE showing similar ratios of drug and metabolite. Patients 1 and 6 were not sampled for serum as they had already received TACE and were awaiting resection when recruitment started.

4.3.5.2 Hepatic parenchymal protein expression and serum drug concentrations

Patients were divided into 2 groups (high and low) based on median microsomal protein expression (quantified by Western blotting) to try and identify possible cut-offs. Patients with low CES-1 showed no difference in SN-38:irinotecan ratio to those with high CES-1 (0.40 vs. 0.37, p=0.91) (Figure 51). Patients with high CES-2 had a non-significant increase in the ratio of SN-38:irinotecan (0.55 vs. 0.23, p=0.11) whilst patients with high CYP3A4 had a non-significant increase in APC:irinotecan ratio (0.58 vs. 0.33, p=0.2). Patients with high UGT1A1 expression had a non-significant trend towards an increased SN-38G:SN-38 ratio (6.78 vs. 2.35, p=0.05).
Figure 51 Box and whisker plots of serum drug and metabolite concentrations stratified by microsomal protein expression quantified by iTRAQ (High = > median, Low = < median). Dark horizontal line = median, red box = interquartile range, whiskers = minimum & maximum value. (A) Patients with low CES-1 showed no difference in SN-38:irinotecan ratio to those with high CES-1 (0.40 vs. 0.37, p=0.91). (B) Patients with high CES-2 had a non-significant increase in the ratio of SN-38:irinotecan (0.55 vs. 0.23, p=0.11). (C) Patients with high UGT1A1 expression had a non-significant trend towards an increased SN-38G:SN-38 ratio (6.78 vs. 2.35, p=0.05) whilst patients with high CYP3A4 (D) had a non-significant increase in APC:irinotecan ratio (0.58 vs. 0.33, p=0.2).
4.3.6 Microsomal metabolism of irinotecan

4.3.6.1 Microsomal metabolite production

Incubations were performed to compare the metabolic activity of each relevant pathway in the metabolism of irinotecan. Microsomal fractions were used as they are enzyme enriched, maximizing drug turnover. Microsomal incubations demonstrated wide variation in drug metabolism (Table 31, Figure 52). SN-38 varied 4-fold from 0.52-2.19 pmol mg\(^{-1}\) min\(^{-1}\). APC was much lower, varying 2.4 fold from 0.1-0.24 pmol mg\(^{-1}\) min\(^{-1}\). UGT1A1 varied 2-fold, from 0.3-0.59 pmol mg\(^{-1}\) min\(^{-1}\). Relative amounts of SN-38G to SN-38 varied from 26.2-81.6%. For both APC and SN-38G, the amount of analyte on column for this level of drug was close to the nominal lower limit of quantification for the assay (See section 3.3.3.4).

<table>
<thead>
<tr>
<th>Patient</th>
<th>SN-38 (pmol mg(^{-1}) min(^{-1}))</th>
<th>APC (pmol mg(^{-1}) min(^{-1}))</th>
<th>SN-38G (pmol mg(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>1.13±0.24</td>
<td>0.14±0.02</td>
<td>0.46±0.04</td>
</tr>
<tr>
<td>Patient 2 #</td>
<td>1.12±0.18</td>
<td>0.21±0.02</td>
<td>0.59±0.04</td>
</tr>
<tr>
<td>Patient 3 #</td>
<td>0.60±0.14</td>
<td>0.23±0.02</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>Patient 4</td>
<td>1.11±0.16</td>
<td>0.24±0.04</td>
<td>0.55±0.07</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.89±0.13</td>
<td>0.16±0.01</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>Patient 6</td>
<td>2.19±0.27</td>
<td>0.22±0.05</td>
<td>0.58±0.04</td>
</tr>
<tr>
<td>Patient 7 #</td>
<td>0.76±0.10</td>
<td>0.10±0.02</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>Patient 8</td>
<td>0.91±0.09</td>
<td>0.19±0.04</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>Patient 9</td>
<td>0.93±0.13</td>
<td>0.20±0.04</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td>Patient 10</td>
<td>0.52±0.10</td>
<td>0.14±0.02</td>
<td>0.42±0.03</td>
</tr>
</tbody>
</table>

Table 31 Microsomal drug metabolism (±SD) (n=3 separate incubations)

#=UGT1A1*28 6/7 heterozygotes
Figure 52 Column chart showing amount of metabolite present after microsomal incubation of 500 nM irinotecan for 120 minutes. (Column = mean of 3 incubations, bars = SD).

There was a positive correlation between the amount of SN-38 present in an incubation and the absolute amount of SN-38G ($r^2=0.67$, $p=0.03$). There was also a clear negative association between the amount of SN-38 present and the relative proportion of SN-38 glucuronidated ($r^2=0.64$, $p=0.04$) suggesting that the glucuronidation pathway has relatively low capacity (Figure 53).
Figure 53 Scatter plots showing relationship between microsomal SN-38 and absolute and relative SN-38G. As SN-38 increases, so does the presence of SN-38G. However, relative glucuronidation decreases with increasing SN-38 suggesting a relatively low capacity pathway.

4.3.6.2 Hepatic parenchymal expression and microsomal drug production

The presence of SN-38 was clearly related to microsomal CES-2 expression ($r^2=0.95$, $p<0.001$) (Figure 54). No correlation was found between APC and CYP3A4 expression ($r^2=0.05$, $p=0.53$) or SN-38 and CES-1 expression ($r^2=0.08$, $p=0.45$) using either WB or iTRAQ. There was no correlation between expression of UGT1A1 and amount of SN-38 ($r^2=0.25$, $p=0.15$) suggesting that inactivation of SN-38 by glucuronidation was unlikely to have an effect of the availability of SN-38 at physiologically relevant doses. There was a strong relationship between the glucuronidation of SN-38 to SN-38G and UGT1A1 expression ($r^2=0.75$, $p=0.01$).
Figure 54 Scatter plots showing correlation between microsomal drug metabolism and microsomal protein expression quantified by Western blot. SN-38 was clearly related to microsomal CES-2 expression ($r^2=0.95$, $p<0.001$). There was also a strong relationship between the glucuronidation of SN-38 to SN-38G and UGT1A1 expression ($r^2=0.75$, $p=0.01$).
4.3.7 Correlation between metabolic profile and pathological tumour response

4.3.7.1 Serum concentration and pathological response

Absolute serum concentration of SN-38 at 1 and 6 hours showed no relationship with proportion of viable tumour ($r^2=0.003$, $p=0.88$), degree of necrosis ($r^2=0.22$, $p=0.24$) or fibrosis ($r^2=0.07$, $p=0.54$). The lack of correlation between pathological tumour response and serum absolute or relative levels of SN-38 suggests single time point assessment of serum metabolites is a poor predictor of local tumour destruction.

4.3.7.2 Protein expression and pathological response

The association between parenchymal protein expression and pathological response was weak. However, microsomal expression of CES-2 and fibrosis showed a clear trend that approached statistical significance ($r^2=0.3948$, $p=0.052$) (Figure 55).
Figure 55 Scatter plot showing relationship between hepatic parenchymal expression of CES-2 and tumour fibrosis after neoadjuvant DEBIRI-TACE.

4.3.7.3 Microsomal production and pathological response

A highly significant association between microsomal SN-38 and degree of tumour fibrosis was observed ($r^2=0.41$, $p=0.01$), with a moderate correlation between microsomal SN-38 and degree of tumour necrosis ($r^2=0.40$, $p=0.07$). There was no correlation between SN-38 and overall tumour viability ($r^2=0.001$, $p=0.48$) (Figure 56).
Figure 56 Scatter plots showing correlation between microsomal SN-38 and validated pathological markers of tumour response (viable tumour, necrosis and replacement by fibrosis).
4.4 Discussion

The purpose of the experiments described in this chapter were to assess the variation in expression of key enzymes involved in the disposition of irinotecan across a panel of 10 patients treated with neoadjuvant DEBIRI-TACE and to relate this variation to differences in metabolic profile. The dataset was then further analysed to investigate the relationship between this metabolic profile and clinical outcome.

Targeted proteomic analysis using Western blot and iTRAQ showed wide variation between patients in the expression of key proteins involved in the metabolism of irinotecan. Previous studies have suggested a 15-fold variation in cytosolic expression of CES-2, with a 3 fold variation in microsomal expression (Xu et al., 2002) as well as two-fold variation in the hepatic expression of CYP3A4 (Wolbold et al., 2003). The current study demonstrated similar variations in expression of CES-2. However, variation in expression of CYP3A4 assessed by Western blotting demonstrated a much smaller range. In contrast, quantification using iTRAQ demonstrated fold-change in keeping with previous reports. The discrepancy may be explained by the relative specificity of each approach. CYP3A4 shares >80% sequence homology with CYP3A43, CYP3A7 and CYP3A5 (Redlich et al., 2008). Similarly, UGT1A1 shares 73% amino acid sequence identity with other UGT1As (Yang et al., 2012). Conversely, sequence homology between CES-2 and CES-1 is only 46% (Holmes et al., 2010). These similarities may explain the fact that quantification by Western blotting and iTRAQ do not precisely mirror each other.

Existing evidence surrounding the relationship between UGT1A1 genotype and drug efficacy and toxicity is conflicting. The UGT1A1*28 6/7 polymorphism has been reported in around 39% of Europeans, with 13% having the 7/7 SNP (Liu et al.,
This study found comparable incidence in a relatively small cohort, with 30% carrying the 6/7 SNP. In this series, 6/7 heterozygotes demonstrated no difference in UGT1A1 protein expression quantified by either Western blot or iTRAQ. Unsurprisingly, there were no differences in serum concentration of SN-38G between 6/6 and 6/7 patients, nor were there any differences in microsomal drug metabolism. These findings suggest that for the dose of irinotecan delivered during DEBIRI-TACE the presence of a UGT1A1*28 SNP does not affect treatment efficacy. Systemic toxicity after the administration of irinotecan normally manifests as diarrhoea and neutropenia, presumably caused by the accumulation of SN-38 in the gut and bone marrow. The UGT1A1*28 SNP has been implicated in higher rates of toxicity after systemic delivery (Iyer et al., 1998). However, there were no instances of irinotecan toxicity in the PARAGON II trial (see section 2.3.2), with similar findings reported in previous cohort studies. It therefore seems unlikely that UGT1A1*28 SNP is associated with increased toxicity after DEBIRI-TACE, presumably because of the relatively low dose of irinotecan delivered during embolisation. However, further large prospective studies are required to definitively answer this question.

Analysis of serum drug concentrations allowed broad assessment of systemic exposure to drug and metabolites after treatment. A correlation between the dose of irinotecan administered and exposure to both irinotecan and SN-38 has been suggested in previous pharmacokinetic studies (Rouits et al., 2008) and although the current study did not perform formal pharmacokinetic analysis, there was a clear correlation between dose of DEBIRI and serum concentration of irinotecan 1 and 6 hours post-TACE. The lack of correlation between SN-38 concentration and delivered dose suggests significant differences in patient drug metabolism. Serum drug and metabolite concentrations did not show any relationship with degree of tumour fibrosis or hepatic parenchymal metabolism, suggesting that assessment of
drug activation by measurement of serum drug concentration at a single time point is a poor predictor of hepatic drug metabolism. However, when serum concentrations were divided into 2 groups based on parenchymal protein expression there was a non-significant trend towards increased serum concentration in patients expressing high levels of the relevant protein for each key metabolite, suggesting that it may be possible to approximate hepatic irinotecan metabolism by measuring peripheral levels of drug metabolites. More comprehensive serum sampling strategies would offer further insight.

At a microsomal level, CES-2 expression showed a very strong correlation with amount of SN-38 confirming that this is the key hepatic enzyme in this activation step. Expression of CYP3A4 did not impact on microsomal activation of irinotecan, suggesting saturation of the CES-2 pathway at physiologically relevant doses. Increasing concentrations of SN-38 led to increasing concentrations of SN-38G. In each case the amount of SN-38G was consistently less than the amount of SN-38 (26.2-81.6%). This contrasts with the serum drug concentrations where SN-38G was consistently 3-4 times higher than SN-38. The relative conversion of SN-38 to SN-38G (as defined by ratio of SN-38G:SN-38) showed a negative correlation with the concentration of SN-38, suggesting that within the microsomal model system this remains a low capacity system that cannot limitlessly increase glucuronidation as required. The ability of hepatic microsomes to activate irinotecan into SN-38 clearly correlated with tumour replacement by fibrotic tissue, suggesting that local drug activation in surrounding hepatic parenchyma is the key step in the effectiveness of DEBIRI with CES-2 the key enzyme in this pathway.

Assessing the relationship between drug metabolism and pathological response within the PARAGON trial is limited by a number of factors. Pathological response was assessed using a relatively crude scoring system. This system has been validated in large series of resected CRLM. In these large series, any potential
sampling or scoring errors would have minimal effect on overall results. By contrast, in a small series of 10 patients under- or over-assessing tumour response could lead to marked distortion in results. The pathological tumour response assessment did not assess location of beads within lesion, proximity of beads to tumour and embolisation pattern – for example, central vs. peripheral embolisation. There also remains the possibility that inherent differences in tumour genotype and phenotype may vary response to DEBIRI. A global proteomic assessment of resected tumour is currently being undertaken using iTRAQ. However, this post hoc analysis is limited by selection bias. Tumour tissue that remains is likely to have a DEBIRI-resistant phenotype, with sensitive tumour tissue likely to have been destroyed during embolisation.

In conclusion, these data show that there is large inter-patient variability in the hepatic expression of key irinotecan metabolizing enzymes particularly CES-2 which is responsible for the formation of the active component SN-38. These wide variations clearly correlate with the ability of hepatic microsomes to activate irinotecan, and suggest patients who are better able to activate irinotecan within normal tissue surrounding tumour achieve better rates of tumour response after locoregional DEBIRI. The data in this small pilot study provide preliminary evidence for the use of hepatic metabolic profiling as an approach to predict response to locally delivered irinotecan chemotherapy, and suggests a pharmacological rationale for whole lobe embolisation for CRLM.
5 Concluding Discussion
Chemotherapy before surgery is an integral part of the treatment algorithm for patients with colorectal liver metastases. For patients with irresectable disease, chemotherapy offers the only opportunity to bring patients to potentially curative surgery. For patients with high-risk resectable disease, chemotherapy offers a test of tumour biology as well as treating occult disease reducing the risk of recurrence therefore improving long-term outcome.

The safety of neoadjuvant systemic chemotherapy before surgery has been demonstrated in a number of studies (Wein et al., 2003; Taleb et al., 2005; Gruenberger et al., 2008; Nordlinger et al., 2008). However, the safety of neoadjuvant DEBIRI has not previously been shown. The PARAGON II trial reported in this thesis clearly demonstrated the safety and feasibility of delivering locoregional neoadjuvant DEBIRI prior to surgical resection. As well as the low rates of peri-procedural morbidity, no patients demonstrated any manifestations of systemic irinotecan toxicity. Although systemic triplet chemotherapy has been shown to increase response rate and overall survival, its use has been limited by high rates of toxicity. A phase III study comparing FOLFIRI vs. FOLFOXIRI demonstrated a doubling in the number of toxicity-related treatment breaks in the FOLFOXIRI arm, as well as increases in neurotoxicity and neutropenia (Falcone et al., 2007). The absence of chemotoxicity reported in the PARAGON II trial suggest that the addition of DEBIRI may allow the delivery of systemic FOLFOX with liver-targeted irinotecan allowing synergistic activity to occur at the site of the greatest tumour burden without overlapping systemic toxicity.

This trial also demonstrated no compromise in surgical safety for liver resection after treatment with DEBIRI, with low post-operative morbidity and no increase in post-operative mortality directly attributable to treatment. Irinotecan-induced CALI is clearly associated with post-operative mortality and the reassuringly low rates of
background liver injury reported in this series suggest that single treatment DEBIRI
do not increase the incidence of liver injury.

The pathological response rates reported after a single treatment are impressive,
with 76% of lesions showing a major or complete pathological response. These
results compare favourably with response rates seen after multiple cycles of
systemic cytotoxic and targeted biological therapies reported by other groups (See
table 32). However, it is important to recognize that patients selected for PARAGON
II will by definition have relatively low-risk tumour biology. All patients had limited
intrahepatic lesions and were deemed not to require neoadjuvant therapy by a
specialist liver MDT. By contrast, most published series reporting pathological
response rates include a mix of initially irresectable lesions, as well as oncologically
high-risk patients in whom a course of preoperative therapy was deemed
necessary. The response rates to DEBIRI may therefore represent a subgroup with
high levels of chemo-susceptibility as well as the effectiveness of treatment.

It has been suggested that larger lesions have inadequate vascular supply to
maintain viable tumour and that some tumour necrosis and fibrosis may be
explained by inadequate perfusion rather than the effect of chemotherapy
(Poultsides et al., 2012). The evidence supporting this hypothesis is conflicting, with
Blazer et al suggesting that tumour size >3cm was an independent predictor of
major/complete pathological response (Blazer et al., 2008). By contrast Rubbia-
Brandt et al (Rubbia-Brandt et al., 2007) found no correlation between size and
response, although there were no complete responses in tumors ≥2 cm.
Table 32 Pathological response rates reported after neoadjuvant treatment with systemic chemotherapy (Blazer et al., 2008). Lesions treated with a single treatment with DEBIRI-TACE have a superior complete and major pathological response rate to lesions exposed to multiple cycles of systemic therapy.

The PARAGON II study reported a median lesion size of 21mm (range 4-150) and although there was a statistically significant difference in median lesion size between minor (18mm) and major response (40 mm), and major and complete (19mm), the fact that the major response group tended to have larger lesion size than the complete response group makes it unlikely that pathological response after DEBIRI-TACE can be explained by size alone. In this study, three patients with lesions greater than 2cm exhibited a complete pathological response and it may be that the targeted mechanism of delivery of DEBIRI-TACE allows it to better achieve high rates of pathological response in large lesions.

The intra-operative identification of previously untreated lesions allows direct assessment of tumour response attributable to DEBIRI. The large amount of viable
tumour observed in lesions supplied by arterial flow proximal to the point of bead release and the absence of tumour in previously unidentified lesions targeted by lobar embolisation supports the hypothesis that tumour fibrosis and necrosis is directly attributable to treatment with DEBIRI, and provides an oncologic rationale for very proximal whole lobe embolisation. These results suggest that undetected micrometastatic lesions within a lobar embolisation zone could be as effectively treated as lesions that are identified preoperatively and more selectively embolised. This finding is important for the design of future trials with lobar treatment with irinotecan bead being effective at destroying occult micrometastases.

Importantly, radiological assessment of tumour response 4 weeks after treatment did not reflect degree of tumour destruction or correlate with long-term outcome. Similar findings have been reported after treatment with other locoregional embolic therapies (Kennedy et al., 2013). However, these findings are in contrast to previously reported series of DEBIRI. A large US led registry of heavily pretreated patients with colorectal liver metastases treated with DEBIRI reported a 3-month RECIST tumour response rate of 65% with 12% of patients showing complete response and 53% showing partial response (Martin et al., 2009), whilst an Italian phase II randomized study reported 69% of patients having complete or partial response after a median of 2 cycles of DEBIRI, compared to 20% in those treated with systemic FOLFIRI. This increased response rate clearly correlated with improved progression free survival (Fiorentini et al., 2012). One possible explanation of the apparently contradictory findings of these studies and the current study may be the time period between treatment and radiological assessment. Embolisation leads to tumour infarct, after which lesions become ischaemic and oedematous which may cause an apparent increase in size. Over time, this oedema and inflammation settles with characteristic tumour replacement by fibrotic tissue (Poultisides et al., 2012) followed by centripetal contraction leading to a reduction in
tumour volume (Ng et al., 2008). Early imaging may therefore capture the swollen oedematous phase of the tumour response that occurs prior to eventual fibrotic involution. These findings have clear clinical implications for the design of any future trials involving DEBIRI where early assessment of tumour response is used to guide management. Contemporary trials investigating the treatment of irresectable colorectal liver metastases have traditionally administered between 4 and 8 cycles of therapy followed by radiological assessment of response (Folprecht et al., 2010; Garufi et al., 2010). For systemic treatments, this assessment may take place within 2 weeks of the final cycle of therapy. The future role of DEBIRI is likely to be alongside systemic therapy for liver-dominant disease, and it is therefore important that any trial design involves early administration of DEBIRI followed by multiple cycles of systemic therapy to allow adequate time for tissue inflammation to settle and to allow a more accurate assessment of tumour response.

Despite relatively short follow up after resection, median DFS was 379 days with a nominal 1-year DFS of 56%. Although this may appear high, meta-analysis has demonstrated that around 65% of patients undergoing hepatectomy for CRLM will develop disease recurrence within 2 years (Jones et al., 2012). The targeted administration of DEBIRI within the PARAGON II protocol meant that tissue exposed to treatment was then resected. Occult disease within the future liver remnant was therefore not treated, and it is therefore unsurprising that rates of recurrence remain in keeping with those reported in chemonaïve patients.

Multivariate analysis of PARAGON II patients identified only KRAS status as a prognostic marker for long-term outcome after resection. The prognostic importance of KRAS status in colorectal cancer is controversial and the data often contradictory (Umeda et al., 2012; Lee et al., 1996; Stremitzer et al., 2012). However, the RASCAL II multicentre study assessed over 3000 patients with all stages of colorectal cancer and demonstrated that those with a KRAS mutation had an
increased risk of recurrence and death (Andreyev et al., 2001) whilst the MSKCC group recently reported a trend towards reduced overall and disease free survival for patients undergoing resection of KRAS mutant colorectal liver metastases (Kemeny et al., 2012). In the current study, KRAS mutation was independently associated with worse DFS. Pathological response showed no relationship with KRAS status, suggesting that KRAS has a prognostic but not predictive role in this cohort. However, 3 patients could not be KRAS typed because of lack of viable tumour after treatment and this remains a potential source of bias. No BRAF mutations were identified in this study. Previous series that have reported very low rates of BRAF resection in patients with colorectal liver metastases that come to resection (Andreou et al., 2012) compared to primary colorectal cancers (Vakiani et al., 2012), suggesting BRAF mutant tumours have more aggressive biology and once metastasised are unlikely to become resectable. Future treatment approaches are likely to rely heavily on the direct analysis of tumour biology to define the optimal treatment approach. Although concordance between primary and metastatic tumour is high for many recognized mutations (PI3KC, RAS, BRAF) (Vakiani et al., 2012), it seems likely that assessment of the metastatic lesion by direct sampling or circulating tumour cell analysis will be necessary to compensate for subtle changes in phenotype between primary and metastatic tumour (Gerlinger et al., 2012).

As will as the development of prognostic markers to identify patients most likely to benefit from surgical intervention, there is growing interest in predictive biomarkers to guide the optimal chemotherapeutic approach. These markers will help to identify patients who will respond well to cytotoxic agents, offering the promise of a stratified treatment approach with increased treatment efficacy whilst minimizing unnecessary toxicity. Tumour expression of genes and proteins involved in the metabolism of chemotherapeutic agents have previously been investigated as predicting benefit from cytotoxic therapies (Kornmann et al., 2003), with TOPO-1
suggested as a potential predictive marker for irinotecan (Braun et al., 2008). There is now growing interest in drug metabolism in surrounding normal tissue as an important factor in defining tumour response.

Irinotecan appears a possible target for such an approach, as conversion into the active metabolite SN-38 is required for the cytotoxic effect. Yu et al demonstrated low levels of CES-2 in colonic cancer compared to normal adjacent colon (Yu et al., 2005), and in vitro modelling has shown the conversion of irinotecan to SN-38 in hepatic tissue (Rivory et al., 1996; Mathijssen et al., 2001) which contains high levels of CES-2 compared to tumour (Guichard et al., 1999). DEBIRI allows targeted delivery of irinotecan directly to liver tissue and tumour, where it achieves high local concentrations (Taylor et al., 2007). It is therefore plausible that the high level of CES-2 in hepatic parenchyma lead to local drug activation that can then diffuse into tumour cells causing death. However, this relationship has never been directly assessed. This study is the first to investigate local activation of drug within normal tissue surrounding tumour as a predictor of response.

Assessment of serum concentrations of irinotecan and SN-38 demonstrated much lower levels than previously reported after intravenous administration of comparable doses of drug (de Jonge et al., 2000; Rouits et al., 2004). De Jonge et al reported a peak irinotecan and SN-38 concentration of 7.6 μM and 130 nM 1.6 hours after the administration of 175-300 mg m⁻² of irinotecan, compared to 188.3 nM and 10.2 nM in this study. The much lower levels of systemic irinotecan detected in PARAGON II likely reflect both the lower dose of drug administered as well as high rates of first-pass metabolism seen after hepatic intra-arterial delivery. This low systemic exposure to irinotecan may provide an explanation for the absence of systemic toxicity seen after treatment with DEBIRI. Interestingly, the relative proportion of irinotecan converted to SN-38 in the current study is much higher than that reported after intravenous administration and suggests that intra-hepatic delivery results in
high levels of drug activation. Liver-directed delivery of irinotecan therefore seems a sensible approach to maximize the activation of irinotecan whilst limiting systemic exposure. Interestingly, wide interpatient variations in serum drug and metabolite concentration were observed after treatment suggesting inherent differences in patient drug metabolism. However, these did not correlate with pathological tumour response or with hepatic drug metabolism. One possible explanation may be that extra-hepatic drug metabolism (e.g. within the gut) alters serum concentrations. Alternatively, $T_{\text{max}}$ may vary between patients because of inherent differences in hepatic metabolic capacity. More formal drug pharmacokinetic modeling would go some way to answering this question.

By contrast, the ability of hepatic microsomes to activate irinotecan into SN-38 clearly correlated with tumour replacement by fibrotic tissue, suggesting that local CES-2 mediated drug activation in surrounding hepatic parenchyma is the key step in the effectiveness of DEBIRI. The hypothesis that fibrosis is the predominant chemotherapy-induced pathologic change in CRLM was first suggested by Rubbia-Brandt et al. (Rubbia-Brandt et al., 2007) and it has since been shown that patients with a large fibrotic response have improved long term outcome (Poultsides et al., 2012). This finding suggests that proximal embolisation, with DEBIRI delivered to a wide area of normal parenchyma rather than tumour tissue, may be a more rational approach to maximise conversion of irinotecan to SN-38 and therefore tumour response.

These results suggest that assessing patients’ hepatic metabolic profile may allow selection of those who are most likely to benefit from DEBIRI. Single serum drug concentrations appear to be a poor surrogate of hepatic metabolism, although there was a non-significant correlation between parenchymal protein expression and serum concentrations suggesting this area may warrant further research. Previous attempts to correlate systemic activation of irinotecan and CES-2 expression in
peripheral blood monocytes have been inconclusive (Cecchin et al., 2005), whilst other proposed markers of hepatic CES-2 activity have produced conflicting results (Guemei et al., 2001; Shingyoji et al., 2004). There is currently therefore no good surrogate for directly assessing hepatic CES-2 expression. Directly assessing hepatic parenchyma is straightforward for patients who are being selected for adjuvant therapy – normal parenchyma will be removed as part of routine liver resection. For patients who are treated with neoadjuvant or conversion intent this is more difficult. One option is liver biopsy, although this is not risk-free (Silva et al., 2008). However, any risk must be balanced against the potential benefits of effective chemotherapy and the avoidance of unnecessary toxicity. The ability to characterise individual drug metabolic profiles may be especially important in patients who have CALI, with both CYP3A and CES-2 pathways impaired in patients with parenchymal liver damage (d'Esposito et al., 2010). Another rational approach may be to induce the expression of CES-2. CES-2 is under the direct control of the transcription factor NRF-2 (nuclear factor erythroid 2-related factor 2), and induction of NRF-2 by butylated hydroxyanisole can increase CES-2 expression (Zhang et al., 2012). Dietary factors such as flavonoids (found in green leafy vegetables) are known to induce NRF-2 in man, suggesting that alterations in diet may increase irinotecan activation (Leonardo et al., 2011). As the amount of drug that can be delivered by beads is directly limited by their loading capacity (Lewis et al., 2006), the ability to maximize the local activation of drug remains an attractive approach to maximize local response. The fully validated bioassay developed as part of this thesis allows the assessment of irinotecan and its metabolites in a number of biological matrices, and this assay is now integral to preliminary animal modelling performed within our group which has demonstrated reduced hepatic irinotecan metabolism within NRF-2 knockout mice, with CES-1 and CES-2 under direct transcriptional control. This work has recently been awarded a large grant from North West Cancer Research.
Future work will be required to identify the precise role of DEBIRI-TACE in the treatment algorithm for stage IV colorectal cancer, with early evidence suggesting that combination treatment alongside systemic chemotherapy (so called FOLFOXDEBIRI) is safe with minimal increase in toxicity compared to FOLFOX alone (Martin et al., 2012). A large multicenter, open-label, two-arm phase III study (PARAGON III) has now been developed to formally assess DEBIRI alongside systemic chemotherapy. The study aims to evaluate whether the addition of DEBIRI to current first line chemotherapy increases the number of patients with initially unresectable colorectal liver metastases converted to resection. The primary endpoint will be conversion to resection, with secondary endpoints to include surgical and chemotherapeutic safety, overall survival and progression free survival. Because of its known prognostic value, patients will be stratified prior to randomization based on KRAS status to ensure equal distribution. The experimental arm (Figure 57) will involve administration of 4 cycles of standard systemic chemotherapy (FOLFOX + Bevacizumab) with 2 lobar treatments with DEBIRI, whilst the control arm will involve 4 cycles of systemic chemotherapy alone. After 4 cycles, patients will be re-imaged and discussed at a specialist liver MDT. Those who have become resectable will be offered surgery, whilst those who demonstrate no response will move to second-line systemic therapy. Patients demonstrating a partial response but who remain unresectable will be offered a further 4 cycles of therapy with 2 further DEBIRI treatments. The study is powered to detect an increase in resectability from 28% to 42%. 490 patients will be recruited to give an 80% power and 5% two-sided significance with a 20% anticipated drop out. Because of work developed as part of this thesis, embolisation will adopt a whole lobe administration strategy to maximize tumour response and treatment efficacy. DEBIRI will also be delivered early in the chemotherapeutic cycle to allow peritumoural oedema to resolve prior to radiologic assessment. PARAGON III will also involve a translational component that will validate hepatic metabolism of
irinotecan as a predictive marker of response using the LC-MS/MS assay developed in this thesis, as well as attempt to develop non-invasive methods of assessment of drug metabolism and response.

In conclusion, the work presented in this thesis clearly demonstrates the feasibility, safety and efficacy of neoadjuvant DEBIRI for the treatment of colorectal liver metastases. It also provides clear evidence that proximal whole lobe chemoembolisation is an oncologically and pharmacologically rational approach. This proximal delivery not only results in destruction of occult micrometastases, but also maximizes the exposure of CES-2 rich normal hepatic parenchyma to irinotecan maximising the interconversion to SN-38 maximising treatment response. These findings have been used to guide the development of a large phase III clinical study, where continued translational work will take place to confirm these findings in a larger randomized patient cohort.
Figure 57 CONSORT diagram for PARAGON III phase III RCT of systemic chemotherapy +/- DEBIRI-TACE for irresectable liver limited metastatic colorectal cancer. Because of the known prognostic value of KRAS, patients will be stratified based on KRAS status to ensure equal distribution between both arms.
Bibliography


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liver metastases treated with neo-adjuvant chemotherapy followed by liver surgery. 


Xu, G., Zhang, W., Ma, M. K. and McLeod, H. L. (2002). Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. Clinical Cancer Research, 8, 2605.


Appendix 1 – Supporting publications & presentations

Published peer reviewed manuscripts


Jones, R.P., Dunne, D., Sutton, P., Malik, H.Z., Fenwick, S.W., Terlizzo, M., O’Grady, E., Koelblinger, K., Stattner, S., Stremitzer, S. Gruenberger, T., Poston, G.J. Segmental and lobar administration of drug-eluting beads delivering irinotecan leads to tumour destruction: a case-control series. HPB 2013; 15(1); 71-7

Peer reviewed manuscripts in press

Jones, R.P., Stättner, S., O’Grady, E., Smhurst, A., Terlizzo, M., Malik, H.Z., Fenwick, S.W., Poston, G.J. Radiological assessment of response to neoadjuvant transcatheter hepatic therapy with irinotecan-eluting beads (DEBIRI®) for colorectal liver metastases does not predict tumour destruction or long-term outcome. European Journal of Surgical Oncology 2013 In press
Published abstracts


Jones, R.P, Malik H.Z., Fenwick, S.W., Byrne, C., Terlizzo, M., O’Grady, E., Stremitzer, S., Gruenberger, T., Poston, G.J. Interim follow up results from a phase II study of neoadjuvant chemoembolization of resectable colorectal liver metastases (CRLM) with transcatheter hepatic therapy with irinotecan-eluting beads. *Journal of Clinical Oncology 2013; 34: Abstract 519*

Jones, R.P, Malik H.Z., Fenwick, S.W., Byrne, C., Terlizzo, M., O’Grady, E., Stremitzer, S., Gruenberger, T., Poston, G.J., Kitteringham, N.R. Prediction of pathologic response of colorectal liver metastases (CRLM) to transcatheter hepatic...
therapy with irinotecan-eluting beads by hepatic activation of irinotecan. *Journal of Clinical Oncology* 2013; 34: Abstract 398

**Oral Presentations**

*British Association of Surgical Oncology Annual Scientific Conference, London, 2010*


*Digestive Disease Federation, Liverpool, 2012*


*International HepatoPancreaticoBiliary Association, Paris, 2012*

**Jones, R.P.,** Sutton, P. Gruenberger, T., O’Grady, E., Terlizzo, M., Malik, H.Z., Fenwick, S.W., Poston, G.J. Precision neoadjuvant transarterial chemoembolisation for colorectal liver metastases; update to a phase II study.

- Plenary session

*Liverpool & Northwest Society of Surgeons, Liverpool 2012*

Appendix 2 - Measurement of Concentrations of Irinotecan and its Metabolite SN-38 in Human Plasma Samples

Preclinical data provided by Biocompatibles
Measurement of Concentrations of Irinotecan and its Metabolite SN-38 in Human Plasma Samples Originating from a Clinical Study to Investigate Transarterial Chemoembolisation (TACE) using Irinotecan Loaded DC Bead Microspheres for the Treatment of Unresectable Metastasis to the Liver in Patients with Colorectal Cancer (Sponsor’s Reference CA1011)
Summary

Plasma concentrations of irinotecan and its pharmacologically active metabolite, SN-38, were available from 10 patients following intra-arterial administration of irinotecan-loaded DC bead microspheres at doses of 36-143 mg (average 85 mg) irinotecan (target dose of 400 mg irinotecan). Pharmacokinetic analysis of the concentration-time data resulted in the following mean pharmacokinetic parameters;

<table>
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<tr>
<th></th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$\text{AUC}_1$ (ng.h/mL)</th>
<th>$\text{AUC}_{24}$ (ng.h/mL)</th>
<th>$\text{AUC}$ (ng.h/mL)</th>
<th>$\lambda_z$ (1/h)</th>
<th>$t_{1/2}$ (h)</th>
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<tr>
<td>Irinotecan</td>
<td>Mean</td>
<td>194</td>
<td>2$^a$</td>
<td>1510</td>
<td>1520</td>
<td>1680</td>
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<td>SD</td>
<td>124</td>
<td>1500</td>
<td>1050</td>
<td>1040</td>
<td>1200</td>
<td>0.1346</td>
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<tr>
<td>SN-38</td>
<td>Mean</td>
<td>16.7</td>
<td>1$^b$</td>
<td>147</td>
<td>147</td>
<td>281</td>
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<tr>
<td></td>
<td>SD</td>
<td>11.3</td>
<td>99</td>
<td>99</td>
<td>352</td>
<td>0.0238</td>
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</table>

$^a$ Value is the median

$^b$ Calculated as ln2/mean $\lambda_z$

NB Mean values for $\text{AUC}_1$, $\lambda_z$ and $t_{1/2}$ must be treated with caution as data from the majority of the patients did not meet the acceptance criteria defined in Data Processing.

Intra-arterial injection of irinotecan-loaded DC bead microspheres at a nominal dose level of 400 mg irinotecan resulted in a mean maximum plasma concentration of 194 ng/mL irinotecan at a median time of 2 hours post-dose. The mean maximum plasma concentration of the pharmacologically active metabolite, SN-38, was much lower, 16.7 ng/mL at a median time of 1 hour post-dose. Inter-individual variability was greater than 50% for irinotecan (CV% = 63.8%) and for SN-38 (CV% = 67.8%). The mean metabolite ratio (SN-38/irinotecan) was low, 0.10, but as SN-38 is more potent than irinotecan, the metabolite concentrations would be more effective than the irinotecan concentrations.

The terminal half-life of irinotecan in was determined to be in the range 1.6-7.2 hours. The equivalent data for SN-38 were 7.6-8.5 hours.

Sample analysis

Plasma concentrations of irinotecan and SN-38 were measured using an HPLC procedure previously validated at CentraLabS Clinical Research (CentraLabS Clinical Research report number BCP 0252/062564). The bioanalytical method is described in the validation report.

Sample measurements were generated in three successful analytical batches.

QC samples prepared during the previous validation study (CentraLabS Clinical Research BCP0252) were used during this study.
The analytical equipment, instrumentation and reagents used in this study, and as recorded in the raw data, were equivalent to those used during the validation study. Any changes had no impact on the conduct or validity of the study.

**Results**

Following treatment, maximum measured plasma concentrations of irinotecan generally occurred at 2 hours post-dose and in the range 0.08 – 2 hours whereas maximum measured plasma concentrations of the metabolite SN-38 generally occurred at 1 hour post-dose and in the range 1-2 hours.

The lack of sample times between 6 and 24 hours post-dose resulted in a paucity of data for the determination of the terminal half-lives of irinotecan and SN-38. In fact, it was not possible to determine accurately a terminal half-life for SN-38 in 7 of the 10 patients for this reason. Nor was it possible to determine accurately a terminal half-life for SN-38 in 7 of the 10 patients. As a further consequence of the paucity of sample times between 6 and 24 hours post-dose, it was not possible to determine accurately AUC for the majority of the patients (7/10 for irinotecan; 8/10 for SN-38). Therefore the values presented in the tables for mean AUC, λz and t1/2 are intended as guidelines only.

Determination of the ratio of AUC24 SN-38 to AUC24 irinotecan (metabolite ratio, not adjusted for molecular weight) resulted in a mean value of 0.10 and indicated that formation of SN-38 from irinotecan was limited.

At the request of the Sponsor, pharmacokinetic parameters of irinotecan and SN-38 were calculated from plasma concentration-time data up to 6 hours post-dose only. Again, an accurate half-life could not be determined in the majority of the subjects.

**Discussion**

Intra-arterial injection of irinotecan-loaded DC bead microspheres at a nominal dose level of 400 mg irinotecan resulted in a mean maximum plasma concentration of 194 ng/mL irinotecan at a median time of 2 hours post-dose. The mean maximum plasma concentration of the pharmacologically active metabolite, SN-38, was much lower, 16.7 ng/mL at a median time of 1 hour post-dose. In general (7/10 patients), the maximum measured plasma concentrations of irinotecan and its metabolite SN-38, occurred at the same time. In two patients the T_max of SN-38 was earlier than that of irinotecan and in 1 patient it was later. It is possible that the apparent shorter T_max for SN-38 was an artefact resulting from a lack of sample times between 1 and 2 hours post-dose.

The mean metabolite ratio (SN-38/irinotecan) was low, 0.10, but as SN-38 is ca 1000-fold more potent than irinotecan, the metabolite concentrations would be ca 100-fold more effective than the irinotecan concentrations. The low concentrations of SN-38 in plasma relative to irinotecan may have been a consequence of the greater volume of distribution of SN-38 as it is lipophilic compared to the water-soluble irinotecan.

The terminal half-life of irinotecan was determined to be in the range 1.6 – 7.2 hours, which is similar to the reported range of 6-12 hours. However, accurate data were available from 3 patients only, due to the limited number of sample points in the terminal phase of the plasma irinotecan concentration-time curve. In addition, two estimates of the terminal half-life were determined from data up to 6 hours post-dose only and therefore represented a different disposition phase of the plasma concentration-time curve. The terminal half-life of SN-38 ranged from 7.6-8.5 hours (data from 2 patients only) in comparison to a reported range of 10-20 hours.
Conclusion

Intra-arterial injection of irinotecan-loaded DC bead microspheres at a nominal dose level of 400 mg irinotecan resulted in a mean maximum plasma concentration of 194 ng/mL irinotecan at a median time of 2 hours post-dose. The mean maximum plasma concentration of the pharmacologically active metabolite, SN-38, was much lower, 16.7 ng/mL at a median time of 1 hour post-dose. The mean metabolite ratio (SN-38/irinotecan) was low, 0.10, but as SN-38 is ca 1000-fold more potent than irinotecan, the metabolite concentrations would be more effective than the irinotecan concentrations.

The terminal half-life of irinotecan in 3 of the patients was determined to be in the range 1.6-7.2 hours, which is similar to the reported range of 6-12 hours. The equivalent data for SN-38 were 7.6-8.5 hours (2 patients) in comparison to a reported range of 10-20 hours.

Plasma concentrations of irinotecan and its metabolite SN-38 in a patient (Subject 1) treated with irinotecan-loaded DC bead microspheres at a nominal dose level of 400 mg irinotecan
Appendix 3 – Research ethics approval
19 November 2008

Mr Graeme Poston
Consultant Surgeon
Liverpool Supra-Regional Hepatobiliary Centre
University Hospital Aintree
Longmoor Lane, Liverpool
L6 7AL

Dear Mr Poston

Full title of study:  A single arm phase II study of neoadjuvant therapy using irinotecan Bead in patients with resectable metastatic colorectal cancer.

REC reference number:  08/H0206/41

Thank you for your email of 19 November 2008, responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements.

This Research Ethics Committee is an advisory committee to South West Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England
Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.ndforun.nhs.uk.

Notice of no objection must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming no objection or giving grounds for objection, as soon as this is available.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

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Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
• Progress and safety reports
• Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencingroup@nres.npsa.nhs.uk.

REC Reference: 04/MRE06/4  Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project

Yours sincerely

Chris Foy
Chair

Enclosures:  "After ethical review – guidance for researchers" SL- AR2 for other studies

Site approval form

Copy to:  Dr Brenda Hall, Biocompatible UK Ltd