Establishing a genotoxic predictive test of toxicities and response to irinotecan chemotherapy in metastatic colorectal cancer

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ABSTRACT

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Irinotecan is a topoisomerase I inhibitor prescribed to treat metastatic colorectal cancer. Its use is limited by the heterogeneity in its toxicities and clinical response which are currently unpredictable for the most commonly prescribed doses. Presented in this thesis are the design, conduct and mechanistic analysis of the first prospective clinical study performed to assess whether DNA damage induced in peripheral blood lymphocytes (PBLs), following irinotecan exposure, is a predictive biomarker of this drug’s effect.

PBLs were isolated from patients before and after receiving irinotecan based chemotherapy. A predictive test of irinotecan effect may have improved the management in 11 of the 42 patients recruited who experienced severe toxicities and 7 who gained no clinical benefit.

Irinotecan did not induce DNA damage that could be measured using the alkaline comet assay in PBLs in vivo. An ex vivo method was subsequently developed using mitogenic stimulation of PBLs prior to treatment with SN-38 (the active metabolite of irinotecan) to induce DNA damage that could then be detected. Results demonstrated the presence of a wide inter-individual variation in the DNA damage levels. Several factors including intrinsic assay variability, cell cycle disturbance, apoptosis and DNA repair were investigated and purported to account for these variations.

DNA damage did not correlate with the presence of the UGT1A1*28 polymorphism (associated with slow glucuronidation of SN-38) nor with toxicities to treatment. Liquid chromatography-mass spectrometry analysis of metabolites extracted from PBLs, demonstrated that glucuronidation of SN-38 was not occurring ex vivo, thus providing a potential explanation for these absent associations. However, DNA damage was weakly associated with tumour response and significantly correlated with progression free survival (PFS).

In conclusion, there was no evidence that this model predicted normal tissue toxicities to irinotecan treatment; but associations, warranting further investigation, of DNA damage with response and survival were demonstrated.
ACKNOWLEDGEMENTS

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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ACA</td>
<td>Alkaline comet assay</td>
</tr>
<tr>
<td>ALS</td>
<td>Alkali labile sites</td>
</tr>
<tr>
<td>APC</td>
<td>7-ethyl-10-(4-amino-1-piperidino)</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia Rad3-related</td>
</tr>
<tr>
<td>BEV</td>
<td>Bevacizumab</td>
</tr>
<tr>
<td>bFOL</td>
<td>Bolus 5-FU, oxaliplatin and leucovorin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSC</td>
<td>Best supportive care</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CapeIRI</td>
<td>Capecitabine and irinotecan</td>
</tr>
<tr>
<td>CapOx</td>
<td>Capecitabine and oxaliplatin</td>
</tr>
<tr>
<td>CES</td>
<td>Carboxylesterase</td>
</tr>
<tr>
<td>cf</td>
<td>Correction factor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>C\textsubscript{max}</td>
<td>Maximum concentration</td>
</tr>
<tr>
<td>CPT</td>
<td>Camptothechin</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CTC</td>
<td>Common toxicity criteria</td>
</tr>
<tr>
<td>CTCs</td>
<td>Circulating tumour cells</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DACH</td>
<td>Diaminocyclohexane</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenyindole</td>
</tr>
<tr>
<td>ddH\textsubscript{2}O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependant protein kinase</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>DPD</td>
<td>Dihydropyrimidine dehydrogenase</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>dTMP</td>
<td>2’-deoxythymidine-5’-monophosphate</td>
</tr>
<tr>
<td>dUMP</td>
<td>2’-deoxyuridine-5’-monophosphate</td>
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<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>
</tr>
<tr>
<td>Em</td>
<td>Emission wavelength</td>
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<tr>
<td>ERCC2</td>
<td>Excision repair cross-complementing gene 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>Ex</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>Exo 1</td>
<td>Exonuclease 1</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FdUrd</td>
<td>5-fluoro-2'-deoxyuridine</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-5-isothiocyanate</td>
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<tr>
<td>FLH-1</td>
<td>Height of the fluorescence peak</td>
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<td>FL2-A</td>
<td>Area of the fluorescence peak</td>
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<td>FL2-W</td>
<td>Width of the fluorescence peak</td>
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<td>FOLFERA</td>
<td>FOLFIRI plus ZD4054 or placebo</td>
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<tr>
<td>FOLFIRI</td>
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<td>Infusional 5-FU, leucovorin and oxaliplatin</td>
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<tr>
<td>FOLFOXIRI</td>
<td>Infusional 5-FU, leucovorin, oxaliplatin and irinotecan</td>
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<td>FSC</td>
<td>Forward-angle light scatter</td>
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<td>FUrd</td>
<td>5-fluorouridine</td>
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<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>G phase</td>
<td>Gap phase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTSP1</td>
<td>Glutathione S-Transferase π 1</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non-polyposis colorectal cancer</td>
</tr>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HPLC fluorescence detection</td>
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<td>HR</td>
<td>Homologous recombination</td>
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<td>Irinotecan, bolus 5-FU and leucovorin</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Irinotecan and oxaliplatin</td>
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<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point</td>
</tr>
<tr>
<td>LV</td>
<td>Leucovorin</td>
</tr>
<tr>
<td>mCRC</td>
<td>Metastatic colorectal cancer</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium eagle</td>
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</tr>
<tr>
<td>NEB</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Clinical Excellence</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMP</td>
<td>Normal melting point</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NPC</td>
<td>7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]</td>
</tr>
<tr>
<td>NR</td>
<td>Not reported</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung cancer</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transporting polypeptide</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PNACL</td>
<td>Protein and Nucleic Acid Chemistry Laboratory</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PS</td>
<td>Performance status</td>
</tr>
<tr>
<td>QBL</td>
<td>Quantum 724 complete media for primary lymphocyte culture</td>
</tr>
<tr>
<td>R and D</td>
<td>Research and development</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised control trial</td>
</tr>
<tr>
<td>REC</td>
<td>Research ethics committee</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria In Solid Tumours</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>s.d</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SN-38G</td>
<td>SN-38 glucuronide</td>
</tr>
<tr>
<td>SPC</td>
<td>Summary of product characteristics</td>
</tr>
<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>SSC</td>
<td>Side-angle light scatter</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin like modifier</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Trypsin/EDTA</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour, node, metastases</td>
</tr>
<tr>
<td>Topo I</td>
<td>Topoisomerase I</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate glycosyltransferase</td>
</tr>
<tr>
<td>UHL</td>
<td>University Hospitals of Leicester NHS Trust</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WCC</td>
<td>White cell count</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray cross-complementing group 1</td>
</tr>
<tr>
<td>5-FdUMP</td>
<td>5-fluorodeoxyuridinemonophosphate</td>
</tr>
<tr>
<td>5-FdUTP</td>
<td>5-fluorodeoxyuridinetriphosphate</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>5-FUTP</td>
<td>5-fluorouridine triphosphate</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 General Introduction

Cancer is the number one fear for the British public, topping the list over dementia, heart disease and terrorism. One third of people are expected to be diagnosed with a malignancy at some point in their lifetime. Colorectal cancer (CRC) is the second most common cause of cancer related death in the United Kingdom, second only to lung cancer (Cancer Research UK, 2011). In order to ensure improved survival and quality of life for patients with this disease, the research community are endeavouring to develop: better prevention strategies, more sensitive methods for its earlier detection and superior treatments.

5-fluorouracil (5-FU), irinotecan and oxaliplatin are the most important cytotoxic drugs currently prescribed to treat CRC. The use of these drugs is limited by the presence of drug resistance and by the development of toxicities. Treatment already places a huge financial burden on the National Health Service (NHS). This cost is constantly increasing not only as a result of having a greater number of drugs to use; but also due to the fact that patients with CRC are living, and thus requiring ongoing medical input, for longer. There is an unmet need in the clinic as we are unable to predict the likely response to the majority of treatments in terms of efficacy or toxicity for an individual patient. The ultimate goal in oncology is to direct drugs to those patients most likely to benefit and to use suitable alternatives in those unlikely to respond or at high risk of serious toxicities. This would lead to the overall gain of improved survival and quality of life for cancer sufferers and cost savings for the NHS. To this end we have now reached the era of personalised medicine.
1.2 Epidemiology of colorectal cancer

In the United Kingdom the overall lifetime risk of developing CRC is 1 in 20 with almost 40,000 people being diagnosed with this disease each year (Cancer Research UK, 2011). The probability of acquiring CRC increases with age (Jemal et al., 2004) and its prevalence is slightly higher in men than women (ratio 1.2:1) (Parkin et al., 2005).

The 5 year survival rate for all patients in the UK, diagnosed with CRC between 2001 and 2006, was approximately 50% (Cancer Research UK, 2011); however, the overall incidence of, and mortality from CRC has been declining due to: improved diagnostic techniques, better screening, greater public awareness, increased removal of adenomas at colonoscopy, a wider use of adjuvant therapy, the development of new drugs and an increase in surgical resections for metastatic disease. The extent of tumour burden at diagnosis is the single most important arbiter of survival outcome (Tenesa and Dunlop, 2009, Bowel cancer UK, 2011). As with all cancers, the majority of deaths are attributable to the development of metastatic disease. Approximately 20 – 25% of patients present with metastasis at the time of their initial diagnosis and an additional 25 –35% of patients will develop metastasis during the course of their disease (Kindler and Shulman, 2001, Van Cutsem and Oliveira, 2009, Taieb et al., 2005). The prognosis for patients with metastatic CRC remains poor with median overall survival times approaching 24 months and the 5-year survival rate being just 10 – 20 % (Jemal et al., 2009, Kopetz et al., 2009).
1.3 Aetiology of colorectal cancer

The exact aetiology of CRC often remains elusive with the vast majority of cases being sporadic, however a number of contributing factors, outlined below have been identified.

1.3.1 CRC in the presence of underlying disease

Only a small proportion of CRCs are associated with underlying co-morbid conditions. Inflammatory bowel disease in the forms of ulcerative colitis and Crohn’s disease render affected individuals particularly susceptible. Patients with acromegaly, characterised by elevated levels of growth hormone and insulin-like growth factor 1 (IGF-1) also have an increased risk of colorectal adenomas and carcinomas (Jenkins and Besser, 2001). In addition, CRC occurs with increased frequencies in patients who have had surgery requiring an anastomosis between the ureter and bowel (Cairns et al., 2010).

1.3.2 Inherited CRC syndromes

There are also some well described genetic disorders with a very high absolute CRC risk for the individuals affected but again, these only account for a small proportion (3 – 5%) of all CRC cases (Cairns et al., 2010, Foulkes, 2008). These syndromes are extensively described in the medical literature (Gallinger, 2009) and include: hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome), familial adenomatous polyposis (FAP), MUTYH-associated polyposis, Peutz-Jeghers syndrome and juvenile polyposis. Although rare, the study of these syndromes has helped to identify some of the genes that are commonly involved in sporadic CRC (Table 1-1). The majority of
familial CRCs are not caused by these syndromes; instead other, less penetrant gene abnormalities are present.

1.3.3 Sporadic CRC

It has long been understood that in sporadic CRC, molecular alterations accumulate in a fashion that parallels the clinical progression of tumors. These molecular changes often involve the mutational activation of an oncogene coupled with the loss of several tumour suppressor genes (TSG) (Vogelstein et al., 1988). Mutations in four to five genes may be necessary for the development of a malignant tumour (Fearon and Vogelstein, 1990). The most commonly involved genes are outlined in Table 1-1.

Incidence rates of sporadic CRC closely parallel economic development reflecting an association with “westernised” lifestyles. CRC occurs at a higher rate in those with diets rich in meat and fat and low in fibre. Physical inactivity, excess body weight, a centripetal fat distribution and smoking also have an influence on the risk of developing this disease. The increased documented incidence in westernised countries may also be in part due to the availability of better diagnostic and recording tools in these areas (Tenesa and Dunlop, 2009).
Table 1-1. Genes commonly involved in CRC.

<table>
<thead>
<tr>
<th>Gene (frequency affected)</th>
<th>Function</th>
<th>Defect in CRC</th>
<th>Germline mutation syndrome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC (85%)</td>
<td>TSG that controls the WNT signal pathway. It also has a role in cell adhesion, cell migration, apoptosis &amp; chromosomal segregation at mitosis.</td>
<td>Inactivated by mutations leading to activation of WNT signalling &amp; initiation of tumour development. It also causes an enhanced mutation rate through chromosomal instability thus promoting established tumour growth.</td>
<td>FAP</td>
<td>(Fodde et al., 2001, Polakis, 1997, Kaplan et al., 2001, Walther et al., 2008)</td>
</tr>
<tr>
<td>MLH1 MSH 2 MSH 6 (15 – 25%)</td>
<td>DNA single nucleotide mismatch repair genes.</td>
<td>Epigenetic silencing leading to loss of protein expression &amp; the accumulation of oncogenic mutations &amp; tumour suppressor loss.</td>
<td>HNPPC</td>
<td>(Lynch et al., 2008)</td>
</tr>
<tr>
<td>TP53 (35 – 55%)</td>
<td>TSG that encodes a transcription factor regulating downstream target genes involved in cell cycle regulation, DNA repair, apoptosis &amp; angiogenesis.</td>
<td>Inactivating mutations leading to loss of function of wild type protein.</td>
<td>Li-Fraumeni syndrome</td>
<td>(Vogelstein et al., 2000, Baker et al., 1990)</td>
</tr>
<tr>
<td>SMAD4 (10 – 35%)</td>
<td>TSG that is located on chromosome 18q. It is a component of the TGF-β signalling pathway which exerts an inhibitory effect on cell growth in the colorectum.</td>
<td>Loss of heterozygosity of chromosome 18q is the most common cytogenetic abnormality in CRC. The gene is therefore deactivated by homozygous deletion / mutation</td>
<td>Familial juvenile polyposis</td>
<td>(Popat and Houlston, 2005, Thiagalingam et al., 1996)</td>
</tr>
<tr>
<td>KRAS (35 – 45%)</td>
<td>Oncogene that codes for a GDP/GTP binding protein. It facilitates ligand dependant tyrosine kinase growth factor signalling.</td>
<td>Activating KRAS mutations inhibit GTPase leading to activation of RAS/RAF signalling</td>
<td>Cardio-facio-cutaneous syndrome</td>
<td>(Forrester et al., 1987, Bos et al., 1987, Andreyev et al., 1998)</td>
</tr>
<tr>
<td>BRAF (8 – 12%)</td>
<td>Oncogene that encodes a protein kinase that acts as a downstream effector of RAS mediated signalling.</td>
<td>Activation through a valine to glutamic acid amino acid substitution leads to aberrant activation of the MEK-ERK kinase pathway</td>
<td>Cardio-facio-cutaneous syndrome</td>
<td>(Davies et al., 2002, Ikenoue et al., 2003)</td>
</tr>
<tr>
<td>TGFBR2 (20 – 30%)</td>
<td>TSG that encodes the receptor responsible for signalling pathways mediating growth arrest &amp; apoptosis.</td>
<td>Inactivated by frame shift mutation in polyA repeat within TGFBR2 coding sequence in patients with mismatch-repair defects or by inactivating mutation of kinase domain.</td>
<td>Marfan’s syndrome</td>
<td>(Markowitz et al., 1995, Grady et al., 1999)</td>
</tr>
<tr>
<td>PTEN (10 – 15%)</td>
<td>TSG that is an inhibitor of PI3K signalling.</td>
<td>Inactivating mutation promotes PI3K cell-survival signalling</td>
<td>Cowden’s syndrome</td>
<td>(Yin and Shen, 2008)</td>
</tr>
</tbody>
</table>

Adapted from (Markowitz and Bertagnolli, 2009) and (Tejpar et al., 2010). TSG tumour suppressor gene. ERK extracellular signal regulated kinase, MEK mitogen activated protein kinase, PI3K phosphatidylinositol 3-kinase. GDP Guanosine diphosphate GTP Guanosine triphosphate.
1.4 CRC prevention

Although there are some definite risk factors allowing the identification of groups of patients at high risk of developing CRC, in many cases the development of this common disease cannot be predicted. This has led to the introduction of the NHS bowel cancer screening programme that commenced in July 2006 and currently offers screening to men and women between the ages of 60 – 75. The aim of screening is to detect polyp cancers that can be completely excised before they develop into more invasive tumours.

Despite the ever increasing understanding into the aetiology and prevention of this disease, metastatic spread is still set to be a common clinical problem for the foreseeable future. There are many patients with advanced disease for whom the realistic therapeutic goal is control not cure of the disease (section 1.5.3). This emphasises the need for research, not only to focus on prevention, but also treatment.

As the majority of CRC cases occur in older people, there is also a pressing need to ensure that these cancer treatments, historically so often associated with high toxicities, are suitable for more elderly people who often have additional co-morbidities.

1.5 Treatment of colorectal cancer

1.5.1 Staging of CRC

The pathological stage of CRC is important in determining not only the prognosis but also the optimal treatment modality and treatment intent. The most commonly used staging system is the tumour, node, metastases (TNM) model as defined by the American Joint Committee on Cancer which classifies the CRC based on: the depth of
invasion of the bowel wall (T), the extent of regional lymph node involvement (N), and the development of metastatic disease (M) (Sobin and Compton, 2010).

1.5.2 Treatment of non-metastatic (stages I – III) disease

Surgery forms the mainstay of treatment for colon cancer in the absence of metastatic disease (M0). The prognosis of patients with T1, T2 and low risk T3 tumours without lymph node involvement is excellent and as such surgical resection is the sole treatment in this situation. Surgery for rectal cancer is technically more difficult than for colon cancer due to the bony constraints of the pelvis limiting surgical access and thus there is a lower likelihood of achieving widely negative resection margins. In resectable rectal cancer, neoadjuvant radiotherapy with or without chemotherapy is the treatment of choice (Kapiteijn et al., 2001).

The use of adjuvant chemotherapy has now become routine practice in patients with high risk T3 and T4 tumours and those with lymph node involvement (N1-2) (Moertel et al., 1990, Twelves et al., 2005, Quasar Collaborative et al., 2007). Combination treatment with oxaliplatin and a fluoropyrimidine has greater efficacy than a fluoropyrimidine alone (Andre et al., 2004, Andre et al., 2009, Yothers et al., 2011). Phase III studies have failed to demonstrate an improvement in disease free survival if irinotecan is used in the adjuvant setting (O’Connell, 2009, Van Cutsem et al., 2009b, Ychou et al., 2009, Saltz et al., 2007b).

1.5.3 Treatment of metastatic (stage IV) disease

Long term survival is still only possible in a small minority of patients with metastatic CRC; the majority receive systemic treatment with palliative intent. There are several licensed agents proven to increase overall survival in this setting.
1.5.3.1 Fluoropyrimidines

5-flourouracil (5-FU) was the first chemotherapeutic agent shown to improve survival in metastatic CRC. It is a cytotoxic antimetabolite that is similar in structure to the organic base uracil; differing by virtue of a fluorine atom in place of a hydrogen atom at the carbon 5 position of the pyrimidine ring (Heidelberger et al., 1957). It is metabolised by enzymes that usually act on uracil and thymine (Figure 1-1).

Figure 1-1. Intracellular 5-FU metabolism and its modulation by leucovorin.

5-fluorouridine (FUrD) and 5-fluoro-2'-deoxyuridine (FdUrd) are formed by the addition of ribose or deoxyribose respectively. Cytotoxic fluorinated nucleotides including: fluorouridine triphosphate (FUTP), fluorodeoxyuridinemonophosphate (FdUMP) and fluorodeoxyuridinetriphosphate (FdUTP) are subsequently formed by phosphorylation. FUTP and FdUTP exert cytotoxicity by being incorporated into RNA and DNA respectively, thus altering the stability and structure of DNA and RNA and interfering with DNA repair and protein synthesis.

Thymidylate synthase (TS) normally catalyses the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP). The methyl donor for this reaction is 5,10-methylene-tetrahydrofolate (CH$_2$FH$_4$). FdUMP competes with dUMP to form a stable complex with TS and the reduced folate cofactor thus inhibiting its effect. Folinic acid (leucovorin, 5-CHOFH$_4$) increases 5-FU activity by stabilising the binding of FdUMP to TS.

Adapted from (Tannock and Hill, 2005) and (Perry, 2008).
Single agent bolus 5-FU achieves response rates of 5 - 15%. This response can be doubled by modulating the effect of 5-FU with either methotrexate or leucovorin. (Advanced Colorectal Cancer Meta-Analysis Project, 1992, Advanced Colorectal Cancer Meta-Analysis Project, 1994, Leichman et al., 1995). A further increase in tumour response rate is achieved by administering 5-FU as a continuous infusion rather than as a bolus (22% vs 14%) (Advanced Colorectal Cancer Meta-Analysis Project, 1998).

More recently, oral fluoropyrimidine derivatives (e.g. capecitabine and uracil-ftorafur) have been developed. Capecitabine, the most widely used of these oral derivatives, is activated to 5-FU in vivo, initially in the liver and then in the tumour tissue itself (Tannock and Hill, 2005). Capecitabine has at least equivalent efficacy and also improved tolerability when compared to bolus intravenous (IV) fluorouracil plus leucovorin (5-FU/LV) (Van Cutsem et al., 2001, Hoff et al., 2001).

Fluoropyrimidines are now most commonly used in combination with either oxaliplatin or irinotecan. Monotherapy is generally reserved for those with a clinical contra-indication to receiving combination treatment.

1.5.3.2 Irinotecan

The next chemotherapeutic agent shown to prolong survival in metastatic CRC, to be introduced to the clinic, was irinotecan (7-ethyl-10-[4-(l-piperidino)-l-piperidino]carbonyloxy camptothecin, CPT-11, Camptosar). This agent was of special interest as it showed activity in metastatic CRC that was resistant to 5-FU/LV. It is a water soluble, semisynthetic derivative of the plant alkaloid camptothecin (CPT) that exerts its cytotoxicity by being a potent inhibitor of topoisomerase I (topo I). The mechanism of action, pharmacokinetics, pharmacodynamics and pharmacogenetics are described in depth in section 1.6.
Irinotecan was first licensed in the UK in 1998 as a single agent in 2nd line treatment for metastatic CRC. It was subsequently shown that the incorporation of 5-FU derivatives into DNA were multiplied by a factor of 1.5 at 24 hours if CRC cells were exposed to irinotecan prior to the 5-FU in vitro (Guichard et al., 1998). The FOLFIRI regimen (irinotecan dose 180 mg/m² with infusional 5-FU) tends to be better tolerated than irinotecan monotherapy (350mg/m²) (Clarke et al., 2011) and is thus the regimen of choice, being licensed for both first and second line treatment of metastatic CRC (section 1.5.3.5). Irinotecan monotherapy is only routinely prescribed, outside of the trial setting, to patients who are intolerant of 5-FU. Capecitabine and irinotecan combinations are generally more toxic than FOLFIRI and so are only used relatively infrequently (Fuchs et al., 2007). The pivotal trials chronicling irinotecan use in the clinical setting are summarised in Table 1-2.
# Table 1-2: Pivotal studies of irinotecan chemotherapy in advanced colorectal cancer.

<table>
<thead>
<tr>
<th>Study description / acronym</th>
<th>Treatment arms</th>
<th>Patient number</th>
<th>Response rates % (p-value)</th>
<th>Median PFS (months) (p-value)</th>
<th>Median OS (months) (p-value)</th>
<th>Grade 3-4 toxicities (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose finding phase I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary sites: colon, head and neck, lung, pleura</td>
<td>Irinotecan 100 to 750 mg/m²</td>
<td>64 (26 with CRC)</td>
<td>PR 23 in CRC</td>
<td>NR</td>
<td>NR</td>
<td>16</td>
<td>Dose limiting toxicity 350 mg/m² 20 500 mg/m² 33 750 mg/m² 100</td>
</tr>
<tr>
<td><strong>Phase II first or second line</strong></td>
<td>Irinotecan 350 mg/m²</td>
<td>212</td>
<td>18 1st line 18.8 2nd line 17.7</td>
<td>4.2</td>
<td>1st line 12 2nd line 10</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td><strong>Phase III second line after 5-FU failure</strong></td>
<td>Irinotecan 300-350 mg/m² BSC</td>
<td>189</td>
<td>NR</td>
<td>NR</td>
<td>9.2</td>
<td>6.5 (0.0001)</td>
<td>22</td>
</tr>
<tr>
<td><strong>Phase III second line after 5-FU failure</strong></td>
<td>Irinotecan 300-350 mg/m² 5-FU by continuous infusion</td>
<td>133</td>
<td>15</td>
<td>4.2</td>
<td>10.8</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td><strong>Phase III first line treatment</strong></td>
<td>IFL Bolus 5-FU/LV</td>
<td>231</td>
<td>39</td>
<td>7.0</td>
<td>14.8</td>
<td>22.7</td>
<td>53.8</td>
</tr>
<tr>
<td>Irinotecan 125 mg/m²</td>
<td>226</td>
<td>21 (&lt;0.001)</td>
<td>4.3 (0.04)</td>
<td>12.6 (0.04)</td>
<td>13.7</td>
<td>66.2</td>
<td></td>
</tr>
<tr>
<td><strong>Phase III first line treatment</strong></td>
<td>FOLFIRI Continuous 5-FU/LV</td>
<td>199</td>
<td>49</td>
<td>6.7</td>
<td>17.4</td>
<td>44.4</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td>188</td>
<td>31 (&lt;0.001)</td>
<td>4.4 (&lt;0.001)</td>
<td>14.1 (0.031)</td>
<td>25.6</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Study description / acronym</td>
<td>Treatment arms</td>
<td>Patient number</td>
<td>Response rates % (p-value)</td>
<td>Median PFS (months) (p-value)</td>
<td>Median OS (months) (p-value)</td>
<td>Grade 3-4 toxicities (%)</td>
<td>Reference</td>
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</tr>
<tr>
<td>Phase III second line</td>
<td>Irinotecan 125 mg/m$^2$</td>
<td>95</td>
<td>NR</td>
<td>4</td>
<td>9</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Irinotecan 300-350 mg/m$^2$</td>
<td>196</td>
<td>3 (0.54)</td>
<td>9 (0.43)</td>
<td>19</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Phase III first line</td>
<td>FOLFIRI</td>
<td>144</td>
<td>47.2</td>
<td>7.6</td>
<td>23.1</td>
<td>13.9</td>
<td>43.1</td>
</tr>
<tr>
<td>BICC-C study</td>
<td>mIFL</td>
<td>141</td>
<td>43.3 (&gt;0.05)</td>
<td>5.9 (0.004)</td>
<td>17.6 (0.09)</td>
<td>19</td>
<td>40.9</td>
</tr>
<tr>
<td></td>
<td>CapeIRI</td>
<td>145</td>
<td>38.6 (&gt;0.05)</td>
<td>5.8 (0.015)</td>
<td>18.9 (0.27)</td>
<td>47.5</td>
<td>31.9</td>
</tr>
<tr>
<td>Phase II second line</td>
<td>Irinotecan 350 mg/m$^2$</td>
<td>43</td>
<td>11.4</td>
<td>4.0</td>
<td>11.2</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>DaVINCI study</td>
<td>FOLFIRI</td>
<td>42</td>
<td>11.4 (0.99)</td>
<td>6.2 (0.34)</td>
<td>15.4 (0.14)</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

For details of individual regimens refer to original reference.
BSC is best supportive care.
Irinotecan monotherapy is administered every 3 weeks except at the dose of 125 mg/m$^2$ when it is given weekly for 4 weeks every 6.
IFL is irinotecan (125mg/m$^2$), bolus 5-FU and leucovorin weekly for 4 weeks every 6. Modified IFL (mIFL) is given days 1 and 8 every 3 weeks.
FOLFIRI is irinotecan (180mg/m$^2$) combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
CapeIRI is irinotecan (250mg/m$^2$) and capecitabine every 3 weeks.
NR not reported, OS overall survival, PFS progression free survival, PR partial response.
1.5.3.3 Oxaliplatin

Oxaliplatin (trans-l-diaminocyclohexane oxalatoplatinum) was the most recent cytotoxic chemotherapeutic agent, shown to improve outcome in metastatic CRC, to be routinely used in clinical practice. This drug is a third-generation platinum compound.

Platinum based drugs have a planar structure with 4 attached chemical groups; two of which are inert carriers and two of which are available for substitution and reaction with molecules such as DNA to form adducts leading to the formation of intra-strand and inter-strand platinum-DNA cross links. This disrupts the structure of the DNA molecule (Martin et al., 2008) thereby activating several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminates in apoptosis (Siddik, 2003, Koopman et al., 2009).

The first two platinum drugs to be routinely used in cancer treatment were cisplatin and then carboplatin, however these agents did not demonstrate efficacy when treating CRC. Oxaliplatin differs from these 2 compounds in that it possesses a 1,2-diaminocyclohexane (DACH) carrier group and a bidentate oxalate reacting ligand (Tannock and Hill, 2005). Oxaliplatin is cytotoxic to tumour cells (including CRC cells) that are resistant to other platinum based drugs (Burchenal et al., 1977, Tashiro et al., 1989, Mathe et al., 1989), probably as a result of the different 3-dimensional structure that results from the presence of the DACH groups (Rixe et al., 1996).

The response rate to oxaliplatin monotherapy is only about 10% (Machover et al., 1996,) however a synergistic interaction with 5-FU exists. In the first line setting an oxaliplatin / infusional 5-FU combination treatment (FOLFOX) improves progression-free survival (PFS) (median PFS 9.0 versus 6.2 months; P =0.0003) and response rate
(50.7% versus 22.3%; P =0.0001) compared to a 5-FU based regimen alone (de Gramont et al., 2000). Similarly, in those with disease progression following FOLFIRI, FOLFOX demonstrates superiority to 5FU/LV alone with response rates of 9.9% versus 0% and time to progression 4.6 months versus 2.7 months respectively (P < 0.001)(Rothenberg et al., 2003). Combining oxaliplatin with capecitabine (CapOx or Xelox) is comparable to FOLFOX and is thus a regimen commonly adopted in some centres in the UK (Cassidy et al., 2011).

1.5.3.4 Other cytotoxic agents
Although fluropyrimidines, irinotecan and oxaliplatin are the most commonly used agents in this setting, occasionally the use of other cytotoxic drugs may be indicated. Raltitrexed is a thymidylate synthase inhibitor that is licensed in the UK for the palliative treatment of advanced CRC, where 5-FU based regimens are either not tolerated or suitable (for example, in patients who develop cardiotoxicity) (NICE Clinical Guidelines, 2011). In addition, Mitomycin C is an antitumour antibiotic that, when combined with either capecitabine or infusional 5-FU, is an effective third line regimen for metastatic CRC resistant to 5FU and irinotecan (Chong et al., 2005, Price et al., 2004).

1.5.3.5 Sequence of treatment
Patients who receive all three of these commonly used cytotoxic agents (5-FU, irinotecan and oxaliplatin) achieve the best overall survival in metastatic CRC (Grothey et al., 2004, Rothenberg et al., 2003). Approximately 70 – 80 % of patients who progress after first line chemotherapy receive further systemic treatment (Tournigand et al., 2004, Seymour et al., 2007). The optimal sequence in which to administer the
drugs has been the topic for several large multicentre prospective phase III studies summarised in Table 1-3. The clinical challenges in the palliative setting are to maintain quality of life throughout treatment and to ensure that patients remain fit to receive subsequent lines of therapy.

When using overall survival as an endpoint; first line single agent 5-FU/LV followed by second line combination therapy is not inferior to a first line combination; however, staged single agents (i.e. 5-FU then irinotecan) are inferior (Seymour et al., 2007, Koopman et al., 2007). First line combination treatment does have an improved response rate and is therefore the standard treatment for patients with an adequate performance status. Second line treatment is frequently less effective than first line; for patients who receive irinotecan chemotherapy 2nd line, following an oxaliplatin based regimen, the median progression free survival (PFS) is only 2.5 – 4.0 months (Tournigand et al., 2004).

FOLFOX is usually the preferred first line treatment following favourable results in one of the first phase III oxaliplatin or irinotecan combination head to head studies (Goldberg et al., 2004) (Table 1-3). However, these findings need to be interpreted with caution as in this study the irinotecan was administered with bolus instead of the optimal infusional 5-FU. Generally, in terms of efficacy, there is no clearly superior combination regimen (Van Cutsem et al., 2010). It is reasonable to recommend that a treatment preference may be based on the toxicity profiles of the drugs. Gastrointestinal disturbances are more common with FOLFIRI whilst neuropathy is common with FOLFOX. A key point to note is that whilst at a population level the two regimens are comparable; for each individual patient it may be that one treatment
would be better in terms of efficacy and tolerability than the other but currently there is no way of predicting this.

Triple combination treatment with 5-FU/LV, oxaliplatin, and irinotecan (FOLFOXIRI) is limited by high toxicities and has predominantly been researched in the neoadjuvant setting when significant tumour reduction may allow curative surgical resection (section 1.5.3.7).
Table 1-3: Pivotal studies evaluating sequential and combination chemotherapy in advanced colorectal cancer

<table>
<thead>
<tr>
<th>Study description / acronym</th>
<th>Treatment arms</th>
<th>Patient number</th>
<th>Response rates % (p-value)</th>
<th>Median PFS months (p-value)</th>
<th>Median OS months (P-value)</th>
<th>Grade 3 – 4 Toxicities (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>First line phase III study</td>
<td>IFL</td>
<td>264</td>
<td>31</td>
<td>6.9</td>
<td>15</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>FOLFOX</td>
<td>267</td>
<td>45 (0.002)</td>
<td>8.7 (0.0014)</td>
<td>19.5 (0.0001)</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>IROX</td>
<td>264</td>
<td>35 (0.3)</td>
<td>6.5 (&gt;0.5)</td>
<td>17.4 (0.04)</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>First line phase III study</td>
<td>FOLFIRI</td>
<td>164</td>
<td>31</td>
<td>7</td>
<td>14</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>FOLFOX</td>
<td>172</td>
<td>34 (0.6)</td>
<td>7 (0.64)</td>
<td>15 (&lt;0.28)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>First then second line phase III study</td>
<td>1st line FOLFIRI 2nd line FOLFOX</td>
<td>109</td>
<td>56</td>
<td>15</td>
<td>8.5</td>
<td>4.2</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>1st line FOLFOX 2nd line FOLFIRI</td>
<td>111</td>
<td>54 (&gt;0.05)</td>
<td>15</td>
<td>8.0</td>
<td>4.2</td>
<td>20.6</td>
</tr>
<tr>
<td>Phase III first, second and third line combination or sequential study. CAIRO study</td>
<td>Sequential. 1st line Capecitabine 2nd line irinotecan 3rd line CapOx Combination 1st line CapeIRI 2nd line CapOx</td>
<td>410</td>
<td>20</td>
<td>10</td>
<td>5.8</td>
<td>8.7</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>410</td>
<td>41 (&lt;0.0001)</td>
<td>7.8 (0.0002)</td>
<td>17.4 (0.3281)</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>Study description / acronym</td>
<td>Treatment arms</td>
<td>Patient number</td>
<td>Response rates % (p-value)</td>
<td>Median PFS months (p-value)</td>
<td>Median OS months (P-value)</td>
<td>Grade 3 – 4 Toxicities (%)</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>----------------------------</td>
<td></td>
</tr>
<tr>
<td>FOCUS study</td>
<td>1st line 5-FU/LV</td>
<td>710</td>
<td>28 (0.07 †)</td>
<td>6.3 (0.75 †)</td>
<td>13.9 (0.16 †)</td>
<td>Diarrhoea</td>
<td>Neutropaenia</td>
</tr>
<tr>
<td></td>
<td>2nd line irinotecan</td>
<td></td>
<td>28</td>
<td>6.3</td>
<td></td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2nd line irinotecan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1st line 5-FU/LV</td>
<td>356</td>
<td>16 (0.001 *)</td>
<td>4.4 (0.74 *)</td>
<td>15.0 (0.65 †)</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2nd line irinotecan</td>
<td></td>
<td>23 (&lt;0.001 *)</td>
<td>4.8 (0.74 *)</td>
<td>15.2 (0.65 †)</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>combination</td>
<td>356</td>
<td>49 (0.001 *)</td>
<td>8.5 ( &lt;0.001 *)</td>
<td>16.7 (0.01 * )</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>combination</td>
<td>357</td>
<td>57 ( &lt;0.001 *)</td>
<td>8.7 ( &lt;0.001 *)</td>
<td>15.4 (0.26 * )</td>
<td>10</td>
<td>28</td>
</tr>
</tbody>
</table>

For details of individual regimens refer to original reference.

IFL is irinotecan, bolus 5-FU and leucovorin weekly for 4 weeks every 6.
FOLFOX is oxaliplatin combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
IROX is irinotecan and oxaliplatin every 2 weeks.
FOLFIRI is irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
CapeIRI is irinotecan and capecitabine every 3 weeks.
CapOx is oxaliplatin and capecitabine every 3 weeks.

P values in sequential studies refer to comparisons between the same line of treatment.

* Compared to first line 5-FU
† Compared to second line irinotecan

Reference: (Seymour et al., 2007)
1.5.3.6 Targeted therapies

In addition to cytotoxic chemotherapy, monoclonal antibodies directed against the vascular endothelial growth factor receptor (VEGFR) and the epidermal growth factor receptor (EGFR) also improve the outcome of selected patients with metastatic CRC.

1.5.3.6.1 VEGFR inhibition with Bevacizumab

Bevacizumab (Avastin) is a humanised antibody against the vascular endothelial growth factor-A (VEGF-A) which is one of the most important pro-angiogenic proteins (Presta et al., 1997). Bevacizumab in combination with chemotherapy is approved for first or second line treatment of metastatic CRC but is deemed inactive for chemotherapy refractory disease (Chen et al., 2006). Bevacizumab monotherapy is not recommended as it has inferior survival to combination treatment (Giantonio et al., 2007).

Bevacizumab has a different side effect profile to cytotoxic chemotherapy. In a phase IV expanded access study of 1914 patients, serious toxicities due to bevacizumab were rare. Grade 3/4 adverse events of interest included bleeding (3%), gastrointestinal perforation (2%), arterial thromboembolism (1%), hypertension (5.3%), proteinuria (1%) and wound-healing complications (1%). Sixty-day mortality was 3% (Van Cutsem et al., 2009c).

Pivotal trials assessing the use of bevacizumab are outlined in Table 1-4. It is important to note that the reported effect is only modest in some of the more recent large phase III studies. There is evidence that treatment continuation until disease progression may be necessary in order to optimize the contribution of bevacizumab to therapy (Saltz et al., 2008) but phase III trials are ongoing to determine the optimal duration over which to administer this drug.
Table 1-4 Pivotal studies evaluating bevacizumab treatment for metastatic CRC.

<table>
<thead>
<tr>
<th>Study description / acronym</th>
<th>Treatment arms</th>
<th>Patient number</th>
<th>Response rates (p value)</th>
<th>Median PFS (months)</th>
<th>Median OS (months)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>First line phase III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVF2107 study</td>
<td>IFL &amp; placebo</td>
<td>411</td>
<td>34.8%</td>
<td>6.2</td>
<td>15.6</td>
<td>(Hurwitz et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>IFL &amp; BEV</td>
<td>402</td>
<td>44.8% (0.004)</td>
<td>10.6 (&lt;0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second line phase III (previous 5-FU/irinotecan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3200 study</td>
<td>FOLFOX</td>
<td>291</td>
<td>8.6%</td>
<td>4.7</td>
<td>10.8</td>
<td>(Giantonio et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>FOLFOX &amp; BEV</td>
<td>286</td>
<td>22.7% (&lt;0.001*)</td>
<td>7.3 (&lt;0.001*)</td>
<td>12.9 (&lt;0.001*)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BEV</td>
<td>243</td>
<td>3.3%</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First line phase II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three Regimens of Eloxatin evaluation “TREE-1” &amp; BEV “TREE-2” studies b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOLFOX</td>
<td>49</td>
<td>41%</td>
<td>8.7</td>
<td>19.2</td>
<td>(Hochster et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>bFOL</td>
<td>50</td>
<td>20% (NS)</td>
<td>6.9 (NS)</td>
<td>17.9 (NS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CapOx</td>
<td>48</td>
<td>27% (NS)</td>
<td>5.9 (NS)</td>
<td>17.2 (NS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOLFOX &amp; BEV</td>
<td>71</td>
<td>52%</td>
<td>9.9</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bFOL &amp; BEV</td>
<td>70</td>
<td>39% (NS)</td>
<td>8.3 (NS)</td>
<td>20.4 (NS)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CapOx &amp; BEV</td>
<td>72</td>
<td>46% (NS)</td>
<td>10.3 (NS)</td>
<td>24.6 (NS)</td>
<td></td>
</tr>
<tr>
<td>First line phase III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO16966 study</td>
<td>FOLFOX / CapOx</td>
<td>701</td>
<td>38%</td>
<td>8.0</td>
<td>19.9</td>
<td>(Saltz et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>FOLFOX / CapOx &amp; BEV</td>
<td>699</td>
<td>38% (NS)</td>
<td>9.4 (0.0023)</td>
<td>21.3 (0.077)</td>
<td></td>
</tr>
<tr>
<td>First line phase III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BICC-C study</td>
<td>FOLFIRI</td>
<td>144</td>
<td>47.2%</td>
<td>7.6</td>
<td>23.1</td>
<td>(Fuchs et al., 2008, Fuchs et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>IFL</td>
<td>141</td>
<td>43.3% (NS *)</td>
<td>5.9 (0.004 *)</td>
<td>17.6 (0.09 *)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CapelRI</td>
<td>145</td>
<td>38.6% (NS *)</td>
<td>5.8 (0.015 *)</td>
<td>18.9 (0.27 *)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOLFIRI &amp; BEV</td>
<td>57</td>
<td>57.9</td>
<td>11.2</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IFL &amp; BEV</td>
<td>60</td>
<td>53.3 (NS *)</td>
<td>8.3 (0.28 *)</td>
<td>19.2 (0.037 *)</td>
<td></td>
</tr>
<tr>
<td>Phase IV uncontrolled</td>
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<td></td>
<td></td>
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<tr>
<td>Bevacizumab Expanded Access Trial “BEAT” study</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CapOx &amp; BEV</td>
<td>346</td>
<td>NR</td>
<td>10.8</td>
<td>23.0</td>
<td>(Van Cutsem et al., 2009c)</td>
</tr>
<tr>
<td></td>
<td>FOLFOX &amp; BEV</td>
<td>552</td>
<td>11.3</td>
<td>25.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOLFIRI &amp; BEV</td>
<td>503</td>
<td>11.6</td>
<td>23.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monotherapy &amp; BEV</td>
<td>300</td>
<td>8.6</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study description / acronym</td>
<td>Treatment arms</td>
<td>Patient number</td>
<td>Response rates (p value)</td>
<td>Median PFS (months)</td>
<td>Median OS (months)</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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<td>--------------------------</td>
<td>---------------------</td>
<td>-------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>First line phase II</td>
<td>FOLFOXIRI &amp; BEV</td>
<td>57</td>
<td>77%</td>
<td>13.1</td>
<td>30.9</td>
<td>(Masi et al., 2011)</td>
</tr>
</tbody>
</table>

For details of individual regimens refer to original reference.

BEV is bevacizumab.

IFL is irinotecan and bolus 5-FU and leucovorin weekly for 4 weeks every 6.

FOLFOX is oxaliplatin combined with bolus and infusional 5-FU and leucovorin every 2 weeks.

IROX is irinotecan and oxaliplatin every 2 weeks.

bFOL is oxaliplatin and bolus 5-FU and leucovorin weekly for 4 weeks every 6.

FOLFIRI is irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.

CapeIRI is irinotecan and capecitabine every 3 weeks.

CapOx is oxaliplatin and capecitabine every 3 weeks.

FOLFOXIRI is oxaliplatin and irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.

* Study not designed to compare TREE 1 and TREE 2 cohorts.

† Compared to FOLFOX.

‡ Compared to FOLFIRI.

§ Compared to FOLFIRI & BEV arm.

NS reported as not significant.
1.5.3.6.2 VEGF inhibition with Aflibercept

Aflibercept is an investigational angiogenesis inhibitor, with a different mechanism of action to bevacizumab, recently demonstrated to be efficacious in the treatment of metastatic CRC. This drug is a fusion protein that binds VEGF, placental growth factor (PlGF) and additional angiogenic growth factors that all play a role in tumour angiogenesis and inflammation. The addition of aflibercept to second line FOLFIRI chemotherapy has been shown to significantly improve response rates (19.8% vs 11.1%; \(P = .0001\)), PFS (6.90 vs 4.67 months; \(P < .0001\)) and OS (13.50 vs 12.6 months; \(P = .0032\)) compared with FOLFIRI alone (Van Cutsem et al., 2011b).

1.5.3.6.3 EGFR inhibition with Cetuximab or Panitumumab

Expression or upregulation of the EGFR gene occurs in 60 – 80% of CRC cases (Cunningham et al., 2004) and is associated with decreased survival (Mayer et al., 1993). Downstream signalling pathways that are activated by the EGFR include the Ras/raf/mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase/Akt pathway. These pathways are important in the regulation of several processes which become uncontrolled in cancer cells including: survival, differentiation, division, migration, invasion, angiogenesis and apoptosis (Ciardiello and Tortora, 2001). Antibodies that bind to the EGRF, blocking signal transduction, can therefore modulate tumour growth.

Cetuximab (Erbitux, a chimeric monoclonal antibody) and panitumumab (Vectibix, a fully human antibody) are two drugs that target the EGFR. Several studies have demonstrated that these agents are effective in combination with chemotherapy in both the first and second line treatment of metastatic CRC. In addition, in contrast to bevacizumab, these antibodies are effective as monotherapy in the treatment of
chemotherapy refractory disease. Cetuximab has also been shown to restore chemosensitivity in patients with irinotecan refractory disease, however this benefit is only small, with the median time to tumour progression being just 4.1 months (Cunningham et al., 2004). It is noteworthy that not all studies have yielded positive results; the COIN study demonstrated an increased response rate, but no evidence of benefit in progression free or overall survival with the addition of cetuximab to first line oxaliplatin combination treatment, even in patients selected by additional mutational analysis of their tumours (see below). The pivotal trials in the development of these EGFR inhibitors in metastatic CRC are summarised in Table 1-5.
Table 1-5: Pivotal studies of EGFR inhibitors in advanced colorectal cancer.

<table>
<thead>
<tr>
<th>Study description / acronym</th>
<th>Treatment arms</th>
<th>Patient number</th>
<th>Response rates % (p value)</th>
<th>Median PFS Months (p value)</th>
<th>Median OS months (p value)</th>
<th>Grade 3-4 Toxicities (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase III 2nd or 3rd line BOND study</td>
<td>Cetuximab</td>
<td>111</td>
<td>10.8</td>
<td>1.5</td>
<td>6.9</td>
<td>2</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>Cetuximab &amp; irinotecan regimen</td>
<td>218</td>
<td>22.9 (0.007)</td>
<td>4.1 (&lt;0.001)</td>
<td>8.6 (0.48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase III second line ERBITUX Plus Irinotecan for Metastatic Colorectal Cancer (EPIC) study</td>
<td>Irinotecan</td>
<td>650</td>
<td>4.2</td>
<td>2.6</td>
<td>10.0</td>
<td>15.7</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td>Irinotecan &amp; cetuximab</td>
<td>648</td>
<td>16.4 (&lt;0.001)</td>
<td>4 (&lt;0.001)</td>
<td>10.7 (0.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase III first line Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer (CRYSTAL) study</td>
<td>FOLFIRI KRAS WT</td>
<td>599</td>
<td>38.7</td>
<td>8.0</td>
<td>18.6</td>
<td>10.5</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>KRAS mutant</td>
<td>350</td>
<td>39.7</td>
<td>8.4</td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>183</td>
<td>36.1</td>
<td>7.7</td>
<td>16.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FOLFIRI &amp; cetuximab KRAS WT</td>
<td>599</td>
<td>46.9 (0.0038)</td>
<td>8.9 (0.0479)</td>
<td>19.9 (0.0419)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>KRAS mutant</td>
<td>316</td>
<td>57.3 (&lt;0.001)</td>
<td>9.9 (0.0012)</td>
<td>23.5 (0.0093)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>214</td>
<td>31.3 (0.35)</td>
<td>7.4 (0.26)</td>
<td>16.2 (0.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase II randomised first line neoadjuvant study CELIM study</td>
<td>FOLFOX &amp; cetuximab</td>
<td>56</td>
<td>68</td>
<td>9.9</td>
<td>33</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>FOLFIRI &amp; cetuximab</td>
<td>55</td>
<td>57 (0.23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study description / acronym</td>
<td>Treatment arms</td>
<td>Patient number</td>
<td>Response rates % (p value)</td>
<td>Median PFS Months (p value)</td>
<td>Median OS months (p value)</td>
<td>Grade 3-4 Toxicities (%)</td>
<td>Reference</td>
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</tr>
<tr>
<td>Phase II first line</td>
<td>FOLFOX</td>
<td>168</td>
<td>36</td>
<td>7.2</td>
<td>18</td>
<td>7</td>
<td>34</td>
</tr>
<tr>
<td>Oxaliplatin and Cetuximab in First-Line Treatment of mCRC (OPUS) study</td>
<td>FOLFOX &amp; cetuximab</td>
<td>169</td>
<td>46 (0.064)</td>
<td>7.2 (0.62)</td>
<td>18.3 (0.91)</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>Phase III first line</td>
<td>Continuous FOLFOX</td>
<td>566*</td>
<td>41</td>
<td>7.9</td>
<td>20.4</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Continuous or intermittent FOLFOX +/- cetuximab</td>
<td>Continuous FOLFOX &amp; cetuximab</td>
<td>49 (0.15)∞</td>
<td>49 (0.31)∞</td>
<td>8.3 (0.31)∞</td>
<td>19.7 (0.67)∞</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>NORDIC VII study</td>
<td>Intermittent FOLFOX &amp; continuous cetuximab</td>
<td>47</td>
<td>47</td>
<td>8.7</td>
<td>22.0</td>
<td>20.3</td>
<td>21.4</td>
</tr>
<tr>
<td>Study description / acronym</td>
<td>Treatment arms</td>
<td>Patient number</td>
<td>Response rates % (p value)</td>
<td>Median PFS Months (p value)</td>
<td>Median OS months (p value)</td>
<td>Grade 3-4 Toxicities (%)</td>
<td>Reference</td>
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</tr>
<tr>
<td>Phase III first line COIN study</td>
<td>Continuous FOLFOX or CapOX KRAS WT KRAS mutant</td>
<td>815 367 268</td>
<td>NR 57 NR</td>
<td>8.1 8.6 NR</td>
<td>15.8 17.9 14.8</td>
<td># 11 15 17 14 # 0 16 &lt;1</td>
<td>(Maughan et al., 2011)</td>
</tr>
<tr>
<td>Phase II first line COIN-B study</td>
<td>Intermittent FOLFOX &amp; cetuximab</td>
<td>77 92</td>
<td>NR 64 (0.049) NR</td>
<td>3.1 6.0 (0.039) NR</td>
<td>17.3</td>
<td>NR NR NR NR</td>
<td>(Wasan et al., 2011)</td>
</tr>
<tr>
<td>Phase III 2\textsuperscript{nd} or 3\textsuperscript{rd} line PICCOLO study</td>
<td>Irinotecan monotherapy</td>
<td>686 in total (460 KRAS WT) 12</td>
<td>NR</td>
<td>NR HR=0.78, p=0.01</td>
<td>NR HR=0.91, p=0.44</td>
<td>NR NR NR NR</td>
<td>(Seymour et al., 2011)</td>
</tr>
<tr>
<td>Phase III first line COIN study</td>
<td>Continuous FOLFOX or CapOX KRAS WT KRAS mutant</td>
<td>815</td>
<td>NR</td>
<td>7.9 (0.98) NR</td>
<td>15.3 20 17 21 14 20</td>
<td># 20 26 31 14 14 20</td>
<td>(Maughan et al., 2011)</td>
</tr>
<tr>
<td>Phase II first line COIN-B study</td>
<td>Intermittent FOLFOX &amp; cetuximab</td>
<td>92</td>
<td>NR</td>
<td>6.0 (0.039) NR</td>
<td>22.8 (NR)</td>
<td>NR</td>
<td>(Wasan et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Continuous FOLFOX or CapOX &amp; cetuximab KRAS WT KRAS mutant</td>
<td>362 297</td>
<td>64 (0.049) NR</td>
<td>8.6 (0.6) NR</td>
<td>17 NR (0.67) 13.6 (0.8)</td>
<td>NR</td>
<td>(Wasan et al., 2011)</td>
</tr>
</tbody>
</table>
For details of individual regimens refer to original reference. Where studies have included KRAS analysis, numbers in bold refer to total number of patients on the specified treatment arm and the italics are the KRAS sub groups. P values are calculated between different treatment groups of the same KRAS status.
FOLFOX is oxaliplatin combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
FOLFIRI is irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
CapOx is oxaliplatin and capecitabine every 3 weeks.
* results for all participants.
BSC is best supportive care, NR is not reported, Wt is wild type, HR is hazard ratio.
IFL is irinotecan, bolus 5-FU and leucovorin weekly for 4 weeks every 6, FOLFIRI is irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
# toxicities were grouped according to whether patients received FOLFOX or CapOx.
∞ p values comparing continuous FOLFOX only versus continuous FOLFOX & cetuximab
In the UK, NICE guidelines currently limit cetuximab use on the NHS to those with potentially resectable liver only metastasis (National Institute for Health and Clinical Excellence (NICE) guidelines, 2009).

Cetuximab and panitumumab provide examples of where biomarker discovery has successfully led to individualised treatment of CRC. Activating mutations in KRAS, a small G-protein downstream of EGFR, have been shown to correlate with poor response to these treatments. Such mutations occur in 30 – 50% of CRC tumours (Karapetis et al., 2008, Amado et al., 2008, Lievre et al., 2008, Van Cutsem et al., 2009a); EGFR inhibitors should therefore only be used in patients whose tumours are wild type for KRAS. More recent efforts to improve individualisation of these treatments have revealed that the efficacy may be further predicted by the presence of additional mutations (Figure 1-2), however these associations are often not straightforward to confirm due to the relatively small frequency with which these mutations may occur (Bokemeyer et al., 2009, Van Cutsem et al., 2011a). This demonstrates that the discovery of robust predictive biomarkers that are both sensitive and specific to change clinical practice is often challenging and unsuccessful.
Figure 1-2. Relationship between biomarkers and response to EGFR monoclonal antibodies in chemorefractory metastatic colorectal cancer. Wt wild type. Mt mutation (Hawkes and Cunningham, 2010).

1.5.3.6.4 Combining biological agents

Preclinical studies showed a synergistic antitumour effect for combining VEGFR and EGFR blockers thus indicating a significant therapeutic potential of this combination strategy (Ciardiello et al., 2000, Tonra et al., 2006). This potential was supported in the BOND2 phase II study in which the patients with irinotecan refractory metastatic CRC receiving the three drugs bevacizumab, cetuximab and irinotecan had an overall survival of 14.5 months (Saltz et al., 2007a). However, in phase III studies, the combining of these biological agents has resulted in increased toxicity and decreased PFS (Hecht et al., 2009, Tol et al., 2009). These combinations are therefore not currently recommended for the treatment of metastatic CRC outside of the clinical trial setting.
1.5.3.7 Neoadjuvant treatment and curative resection of CRC metastasis

Surgery provides the only chance of cure in a limited number of patients with CRC metastasis restricted to the liver or lung. Chemotherapy with either an oxaliplatin or irinotecan based doublet with the addition of cetuximab or bevacizumab may be used to downstage some tumours and increase the resectability (Van Cutsem et al., 2009a, Bokemeyer et al., 2009, Saltz et al., 2008).

There is evidence that the cure rate may be further increased by using higher than standard doses of neoadjuvant irinotecan. This could be of particular relevance to those with a KRAS mutation who do not benefit from the addition of cetuximab in the first line setting. If the dose of irinotecan monotherapy is escalated, 31 – 63% of patients are able to tolerate a dose of 500 mg/m². The main dose limiting toxicities are neutropenia and diarrhoea (Ychou et al., 2002, Van Cutsem et al., 2005). Dose escalation of the irinotecan as part of a FOLFIRI regimen is also feasible in some patients (Mineur et al., 2010). In a first line phase II study, irinotecan at a dose of 260mg/m² was given to patients with metastatic CRC either in combination with the standard 5-FU/LV infusional regimen or in a simplified schedule with the 5-FU bolus on day 1 only. The use of the simplified regimen reduced grade 4 neutropenia from 18% to 12% and grade 3 diarrhoea from 26% to just 10%. The response rate in this study was 57% and secondary resection of metastasis was possible in 28% of patients (Ducreux et al., 2008). However, these findings have not been consistent across all studies; in another phase II study of high dose FOLFIRI, although it was once again confirmed that the high dose combination was feasible, none of the 54 patients treated received a metastatectomy (Duffour et al., 2007). The dose escalation approach probably therefore needs to be reserved for patients with potentially
resectable liver metastasis confined to the liver. The next step optimising neoadjuvant systemic treatment is to combine high dose irinotecan based treatment with either cetuximab or bevacizumab. Such studies are now recruiting (Hebbar et al., 2009) Results with the triple combination FOLFOXIRI are also encouraging. Toxicities (mainly neurotoxicity and uncomplicated neutropenia) are moderately increased but the response rate is high (66%) and further improves the chance of metastatic resection (Falcone et al., 2007, Masi et al., 2009). The addition of bevacizumab to this triplet regimen is also safe and under further investigation (Masi et al., 2011).

1.5.3.8 Treatment of metastatic CRC summary statement

The treatment of metastatic CRC has markedly improved over the past decade but is still mainly palliative in intent. Despite the development of new biological therapies, cytotoxic chemotherapy remains the backbone of treatment. The challenge to the clinician is to maximise clinical response but limit toxicities. Irinotecan is an essential drug in the armoury to treat metastatic CRC. As surgical and pharmacological treatments advance, irinotecan in combination with these other drugs may increasingly play a part in downstaging and so improve the resectability of metastasis and increase cure rates. In the neoadjuvant setting there is evidence that in those who could tolerate irinotecan well, a higher than conventional dose may improve outcome but there is the risk of life threatening toxicities. Other than for EGFR inhibitors, there is no way of predicting response or toxicities to the drugs used to treat CRC. Whilst new drug development is important, it is also vital that studies continue to be conducted to improve the safety, tolerability and efficacy of established drugs to ensure that patients get the full benefits of combination chemotherapy as soon as possible.
1.6 Irinotecan

The information above (section 1.5) illustrates that irinotecan is one of the most important agents used to treat metastatic CRC. However, as illustrated with the successful application of a biomarker in individualising treatment with EGRF inhibitors, irinotecan prescribing could also be improved to maximise response and/or minimise toxicities.

1.6.1 Discovery and development

The origin of irinotecan dates back to the late 1950s when a screening program of natural products was conducted by the United States Department of Agriculture (USDA) (Wall et al., 1966, Wall and Wani, 1996). An extract of Camptotheca acuminata, a plant native to China and Tibet was shown to have cytotoxic antitumour activity. Subsequent studies conducted at the National Cancer Institute (NCI) demonstrated that camptothecin (CPT) was the active constituent. The first animal study demonstrated that CPT was active in the life prolongation of mice treated with L1210 leukaemia cells (Wall et al., 1966).

CPT has a pentacyclic structure (rings A-E) featuring an α-hydroxy-δ-lactone moiety on the E ring, which is the principal requirement for its activity (Wall and Wani, 1977) (Figure 1-3). It is insoluble in virtually all organic compounds except dimethyl sulfoxide (DMSO) in which it exhibits moderate solubility (Wall and Wani, 1996). The first soluble derivative was a sodium carboxylate but this achieved only low response rates and high toxicities (Moertel et al., 1972). It is now known that all CPT derivatives and metabolites are subject to spontaneous inter-conversion between a closed lactone ring (active) and an open carboxylate ring (inactive) form by reversible pH dependant hydrolysis (Wani et al., 1980, Rivory et al., 1996). It was therefore necessary to develop
a soluble analogue able to exist in the active closed ring lactone form. It was determined that number of possible substitutions to CPT could be made and those limited to rings A and B did not affect the rate or extent of conversion of the lactone to inactive carboxylate form (Fassberg and Stella, 1992, Sawada et al., 1996).

Irinotecan, developed jointly by Daiichi and Yakult Honsha in Japan, was the first water soluble CPT analogue to undergo extensive clinical evaluation and obtain regulatory approval.

**Figure 1-3. The chemical structure of irinotecan and its major metabolites.** Adapted from (Perry, 2008).

### 1.6.2 Pharmacokinetics

The metabolism of irinotecan is summarised in Figure 1-4. Irinotecan is a prodrug that is activated by hydrolysis of its dipiperidino moiety to the active metabolite SN-38
(which is 100 – 1000 times more cytotoxic than the parent drug) (Slatter et al., 1997, Mathijssen et al., 2001. This hydrolysis is catalysed by human carboxylesterases (CES) types 1 and 2, which are expressed in normal tissues, predominantly the liver and also in the tumour tissue itself. The affinity for this reaction is low with only 3% of irinotecan being converted into SN-38 (Tsuji, 1991 #414, Wu et al., 2002).

Alternative metabolic pathways exist whereby irinotecan is converted into inactive metabolites 7-ethyl-10-(4-amino-1-piperidino) (APC) and 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] (NPC) by oxidation of the terminal or distal piperidine rings respectively. Cytochrome P4503A (CYP3A), primarily CYP3A4 and CYP3A5 catalyse these reactions (Haaz et al., 1998, Santos et al., 2000) which compete with the formation of SN-38. The pharmacokinetics of SN-38 are therefore influenced by inhibitors or inducers of CYP3A, which lead to a respective increase or decrease in its formation (Kehrer et al., 2002, Mathijssen et al., 2002). NPC may be converted to SN-38 by CES.

Clearance of SN-38 is by biliary glucuronidation to an inactive SN-38 glucuronide (SN-38G) catalysed by the uridine diphosphate glycosyltransferase (UGT) family polypeptides (Atsumi et al., 1991). The UGT gene expresses 9 functional UGT1A proteins by alternative splicing. UGT1A1 is the major UGT1A isoform that conjugates SN-38 (Iyer et al., 1998), although others e.g. UGT1A7 and UGT1A9 are also involved (Carlini et al., 2005).

Renal clearance of irinotecan and its metabolites accounts for < 20% of its excretion. Most of the remaining elimination, particularly of SN-38G, is biliary (Gupta et al., 1997). Membrane transporters are responsible for the uptake of SN-38 from plasma into the hepatocytes (e.g. organic anion transporting polypeptide 1B1 (OATP1B1)
which is encoded by the SLCO1B1 gene) (Nozawa et al., 2005). ATP binding cassette (ABC) pumps remove the drug and its metabolites from the cells. Biliary elimination occurs via several hepatic ABC transporters (ABCB1, ABCC1, ABCC2, ABCC4, ABCG2) (Jansen et al., 1998, Luo et al., 2002, de Jong et al., 2007).

Once biliary excretion of SN-38G, has occurred, bacterial β-glucuronidases may reverse this reaction thus toxic SN-38 can be regenerated in the gut. Several environmental (e.g. co-medications, diet) and genetic factors may affect the expression and function of the numerous proteins that are involved in the metabolism of irinotecan. The pharmacokinetics of this agent and its metabolites therefore varies greatly between individuals.

Figure 1-4. The metabolism of irinotecan
1.6.3  Mechanism of action

SN-38 exerts its cytotoxicity by inhibiting DNA topoisomerase I (topo I) (Hsiang et al., 1985).

1.6.3.1  Topoisomerase I

Topo I is a nuclear enzyme which possesses both DNase and ligase activity in one polypeptide. It plays a fundamental role in solving the complex topological problems encountered during DNA replication, transcription and repair recombination (Wang, 1996).

During the process of DNA replication, topo I acts in four main steps (Pommier et al., 1998). Firstly it binds to double stranded DNA. Next, it forms a covalent adduct by transesterification whereby a tyrosine hydroxyl group of topo I is linked to the 3′ phosphate of a phosphodiester bond (Champoux, 1977). This liberates the 5′ hydroxyl to generate a single strand DNA break (SSB) (Pommier et al., 1998). The covalent enzyme-DNA intermediate is termed the cleavable complex. This third step is single strand passage when the transient DNA break enables the relaxation of the torsionally strained supercoiled duplex DNA. The final step is DNA re-ligation and the enzyme is released.

1.6.3.2  Inhibition of Topoisomerase I by SN-38

SN-38 inhibits the final rejoining step of the topo I reaction thereby trapping the reversible cleavable complex and retaining SSBs (Hsiang et al., 1985, Jaxel et al., 1988). These SSBs lead to the development of more toxic double strand breaks (DSBs) by causing replication fork collapse during attempted DNA replication; ultimately these DNA breaks trigger apoptosis (Hsiang et al., 1989, Ryan et al., 1991) (Figure 1-5).
Even though topo I is expressed throughout the cell cycle (Pommier et al., 1994), SN-38 is most cytotoxic to cells that are in S phase because the development of DSBs is dependent on DNA replication (Strumberg et al., 2000). Apoptosis is not the only response of cells to DSBs; the DNA damage may also be detected by the S-phase checkpoint mechanism and thus G₂ arrest, cellular senescence or repair may ensue (Shao et al., 1999, Covey et al., 1989, Dubrez et al., 1995, O'Connor and Kohn, 1992). There is controversy as to the relative contribution of cell cycle arrest and/or apoptosis to tumour inhibition (Brown and Attardi, 2005).

Figure 1-5. The mechanism of action of irinotecan

The p53 protein plays a vital role in both apoptosis and cell cycle arrest and it has been shown that when CRC cell lines are exposed to irinotecan in vitro, those with wild type p53 experience long term cell cycle arrest whereas apoptosis occurs in the p53 mutants. Apoptosis and cell cycle arrest may therefore be equipotent mechanisms mediating the chemotherapeutic effects of irinotecan (Bhonde et al., 2006).
There is also evidence demonstrating that cytotoxicity does not occur exclusively in S phase, namely: topo I levels and S phase fraction do not predict cytotoxicity, irinotecan is active in some cancers with low S phase fractions and it is still cytotoxic when cell division is inhibited by the addition of aphidicolin *in vitro* (Dubrez et al., 1995, Goldwasser et al., 1995, Goldwasser et al., 1996). Topo I inhibitors act mainly in actively transcribed genes (Zhang et al., 1988), the mechanism of this non S phase cell death is most likely due to transcriptionally mediated DNA damage (Morris and Geller, 1996).

Repair of SN-38 induced DSBs can only occur after the trapped topo I is removed by proteolysis, which occurs when conjugation with ubiquitin marks the topo I for destruction by the 26S proteasome (Desai et al., 1997); a small ubiquitin like modifier (SUMO-1) can also conjugate topo 1 (Mo et al., 2002). Homologous recombination (HR) is the predominant mechanism for the repair of the DSBs that are produced when the replication fork collapses. HR functions during late S and G2 phases of the cell cycle when a homologous template of DNA is available to accurately repair the damage (Bolderson et al., 2009). This process involves the broken duplex invading the DNA double helix of a homologous undamaged partner molecule (the mobile junction formed between the 4 strands of DNA is known as a Holliday junction), the subsequent copying of information, the extension of the DNA by DNA polymerase, Holliday junction resolution and ligation catalysed by DNA ligase (Shrivastav et al., 2008).

### 1.6.4 The efficacy and toxicity of irinotecan in clinical practice.

Irinotecan is now firmly established in the clinic as one of the most important cytotoxic drugs in the treatment of metastatic CRC (section 1.5.3.2). Unfortunately, its use is limited as it has a narrow therapeutic window and large inter-individual differences in
its pharmacokinetics exist. This results in overtreatment with unacceptable toxicities in approximately one third of patients receiving the drug. On the other hand, some patients may be undertreated and so receive a suboptimal therapeutic effect.

Common side effects include bone marrow suppression, diarrhoea, nausea, vomiting, alopecia and fatigue. The diarrhoea may be acute or delayed. Acute diarrhoea typically occurs alongside excessive sweating and abdominal cramps as part of a cholinergic syndrome during or soon after the irinotecan infusion. This can usually be prevented by administering prophylactic atropine. Delayed diarrhoea tends to be more severe and protracted. It is controlled by loperamide in some patients (Abigerges et al., 1995). Delayed diarrhoea and neutropaenia are the most important of the irinotecan induced toxicities and they may be life threatening especially if they occur simultaneously. The toxicity rates from major clinical trials are shown in table Table 1-2.

1.6.5 Pharmacokinetics and pharmacogenetics of irinotecan in clinical practice

There is an inverse relationship between SN-38 glucuronidation rates and severity of diarrhoea; this indicates that inefficient glucuronidation leading to SN-38 accumulation causes toxicities (Gupta et al., 1994). This relationship has also been demonstrated in animal studies (Araki et al., 1993). However, it is not always straightforward; in some individuals the reverse may be true and glucuronidation may increase diarrhoea by directing SN-38G to the gut which may then be reactivated to toxic SN-38 by bacterial β-glucuronidases. Consequently, despite the thorough understanding of the numerous pathways involved in irinotecan metabolism, the pharmacokinetic effect is not always straightforward to predict. Over- or under-expression of several of the genes involved in irinotecan metabolism (section 1.6.2) influence both the absolute levels and overall duration of SN-38 exposure and thus affects the efficacy and toxicity of this agent.
Various methods to modulate irinotecan pharmacology have been investigated with the aim of decreasing its systemic toxicity whilst still maintaining antitumor efficacy. One such method involved administering irinotecan over a prolonged infusion time of 96 hours. The main advantage of this method was thought to be that the prolonged infusion would produce lower peak-plasma drug concentrations, thus saturation of CES would be avoided, leading to more efficient drug activation. In addition, lower peak concentrations of SN-38 could also lead to more efficient hepatic glucuronidation and biliary excretion, thereby reducing systemic toxicity. This was shown to be a feasible regimen in a phase I study (Takimoto et al., 2000).

Other drugs may be used to favourably modulate irinotecan activity. One such example is combining low dose irinotecan with ciclosporin. The rationale behind this combination is that ciclosporin reduces the hepatobiliary clearance of irinotecan and therefore, may be combined with a lower irinotecan dose to give equal efficacy to standard dose monotherapy but with fewer toxicities. This combination has indeed been associated with less diarrhoea as assessed by decreased loperamide use. However, the combination was inferior in terms of efficacy compared to standard monotherapy, thus this regimen cannot be recommended as a standard treatment option (Middleton et al., 2011).

1.7 Biomarkers for personalised colorectal cancer treatment

Biomarkers are defined as “characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathological processes or responses (pharmacologic or otherwise) to a therapeutic intervention”. They have
several roles in the diagnosis and management of cancer patients including (Newell, 2010):

- Pre-disposition biomarkers – identify individuals at risk of developing cancer
- Screening biomarkers – enable early detection of cancer
- Diagnostic biomarkers – enable definition of tumour type, stage and grade
- Prognostic biomarkers – provide information regarding outcome irrespective of therapy
- Predictive biomarkers – Predict response or toxicities to a particular therapy
- Pharmacological biomarkers – demonstrate pharmacokinetics (active drug concentration) and pharmacodynamics (if the drug is reaching its target).
- Surrogate response biomarkers – enable early prediction of ultimate clinical efficacy.

Although there has been a good understanding behind the molecular genetics of colon cancer since the 1980s (Vogelstein et al., 1988), there is still a relative paucity of biomarkers with proven utility in this setting. The most studied biomarkers are somatic (acquired) and usually involve testing of the tumour tissue itself. Several techniques have been investigated including: genotyping, immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and circulating tumour cell (CTC) detection. More recently a gene-signature based on tumour RNA expression has been validated to estimate recurrence risk and benefit from adjuvant FU/LV in stage II CRC (Gray et al., 2011). This is now commercially available as the Oncotype DX Colon Cancer test (Genomic health, 2011). Similarly a DNA microarray based prognostic assay using formalin fixed paraffin embedded samples has also been described (Kennedy et al.,
2011) however TNM staging is still the major prognostic tool currently in widespread use in the clinic.

There was an eight fold rise in publications on predictive biomarkers between 2000 and 2008 demonstrating their current high importance within the clinical research community (Alymani et al., 2010). To date, the only predictive biomarker routinely used for CRC patients is the KRAS gene for EGFR targeted treatment (section 1.5.3.6.2). Potential predictive biomarkers of response for irinotecan include tumour topo I expression (Kostopoulos et al., 2009, Braun et al., 2008) and the presence of microsatellite instability (MSI) (Bertagnolli et al., 2009, Fallik et al., 2003). Germline (inherited) changes may be used to identify pharmacogenetic variations in drug metabolism. Gene variants in the TS and the dihydropyrimidine dehydrogenase (DPD) genes may be associated with toxicities to 5-FU. Polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene are associated with capecitabine toxicities. Polymorphisms in the Glutathione S-Transferase π 1 (GTSP1) gene which facilitates glutathione conjugation are associated with oxaliplatin toxicities (reviewed in (Chua et al., 2010, Ross et al., 2010). Predictive pharmacogenetic biomarkers for irinotecan effect are described in detail in section 1.7.2.

The vast array of biomarkers investigated to date demonstrates that there is a great potential for a personalised approach to chemotherapy in metastatic CRC. However, this is a highly challenging area of research as illustrated by the many initially promising biomarkers that have failed to translate into clinically useful applications.

1.7.1 Why is there a need for a predictive test of irinotecan effect?

Irinotecan is currently prescribed using a patient’s body surface area, at doses derived from clinical trials based on outcomes across populations. This approach does not
account for inter-individual differences in irinotecan pharmacokinetics, therefore inter-patient variability in the response and toxicities to treatment exist. At worst these toxicities may be fatal (Fuchs et al., 2003). Data from the QUASAR adjuvant study in CRC demonstrates that if patients experience significant toxicity their treatment is delayed which therefore reduces the planned dose-intensity and may result in decreased effectiveness of the treatment (Kerr et al., 2000). In patients with metastatic incurable disease it important to ensure that optimal treatment is delivered but not at the expense of unacceptable toxicities and worsening of quality of life. These unpredictable toxicities lead to a proportion of patients choosing not to receive irinotecan and thus potentially being undertreated.

It is highly desirable to identify a particular phenotype or genotype that takes into account each individual’s pharmacologic profile and thus predicts likely drug effect. This would ensure that the dose could be optimised; with a high dose being indicated in those likely to tolerate the treatment well and a dose reduction or a suitable alternative (of the several treatment options available for metastatic CRC) being more appropriate in those in whom it would be overly detrimental. The overall gains would be: a) improved survival by maximising dose intensity and improving the neoadjuvant debulking of potentially resectable metastasis (section 1.5.3.7), b) improved quality of life by avoiding toxicities and c) cost savings for the NHS by reducing hospital admissions. Many groups have attempted to develop such a test and a variety of methods (outlined below) have been investigated.
1.7.2 Predictive tests of irinotecan effect

1.7.2.1 Routine baseline parameters

Baseline characteristics such as a poor performance status and female sex are associated with an increased risk of toxicities (Innocenti et al., 2004, Kweekel et al., 2008b, Innocenti et al., 2009, Cecchin et al., 2009).

Bilirubin levels greater than three times the upper limit of normal are an absolute contraindication to treatment because of the predominantly hepatic excretion of this drug (section 1.6.2). More mild elevations in bilirubin levels have also been associated with toxicities (Freyer et al., 2000, Innocenti et al., 2004, Liu et al., 2008). However, conflicting data have failed to demonstrate any association between baseline bilirubin and irinotecan toxicity or efficacy (Ando et al., 2000, Meyerhardt et al., 2004, Rouits et al., 2004).

1.7.2.2 Use of probe drugs

Use of in vivo probe drugs that mimic the metabolism of cytotoxic drugs has been tried with varying success (Kharasch et al., 2005). No association was demonstrated between erythromycin metabolism and irinotecan clearance (Mathijssen et al., 2004). Erythromycin is, like irinotecan, a substrate for CYP3A4 and ABCB1 however it differs in that it is not metabolised by CYP3A5 (Kinirons et al., 1999). Midazolam however, is metabolised by both CYP3A4 and CYP3A5 but it is a poor substrate for ABCB1 (Kim et al., 1999) and in contrast to erythromycin, its clearance is associated with irinotecan pharmacokinetics ($r=0.745$, $P<0.001$) (Mathijssen et al., 2004).

An individualised dosing equation for irinotecan monotherapy that incorporated CYP3A activity (as measured by midazolam clearance,) height and $\gamma$-
glutamyltransferase activity was developed. This equation was compared to traditional dosing based on BSA in a small randomised study of 40 patients; the interindividual pharmacokinetic variability of irinotecan and SN-38 were 19% to 25% lower in the experimental group (not significant). Using the individualised dosing reduced the incidence of grade 3 and 4 neutropaenia (45% versus 10% p = 0.013) but severe diarrhoea was 10% in both groups (van der Bol et al.).

Similarly, the potential activity of CYP3A4 may be estimated from cortisol biotransformation into 6b-hydroxycortisol (Yamamoto et al., 2000). Patients experiencing neutropaenia or diarrhoea during the first 4 courses of treatment have a 1.84 fold higher urinary 6b-hydroxycortisol/ cortisol ratio following administration of 300 mg of cortisol than those without toxicities (Rouits et al., 2008).

1.7.2.3 Genotyping predictive tests

The comprehensive understanding of the pathways of irinotecan metabolism (section 1.6.2) has allowed identification of many candidate genes.

1.7.2.3.1 Carboxylesterase (CES)

The variability in irinotecan conversion to SN-38 by hydrolysis may theoretically be due to inter-patient differences in CES activity. Unfortunately, estimation of CES activity shows poor correlation to the area under the curve (AUC) of SN-38 or summation of SN-38 and SN-38G (Gupta et al., 1994). Although several polymorphisms in the CES genes are known to exist, no associations with irinotecan toxicity have yet been proven (Smith et al., 2006).

However, contrary to this Ceccin et al demonstrated that CES2 mRNA expression in PBLs is associated with toxicities. Eight of 23 high CES2 mRNA-expressing patients
(34.8%) developed grade 3 to 4 neutropenia or diarrhoea compared with 2 of 22 (9.1%) in the low CES2-expressing group (P = 0.071) (Cecchin et al., 2005). These findings have not been validated.

1.7.2.3.2 Cytochrome P4503A (CYP3A)

High expression of CYP3A leads to reduced formation of SN-38 due to the preferential metabolism of irinotecan to APC and NPC and vice versa. The importance of this enzyme’s effect on irinotecan metabolism is well recognised; the summary of product characteristics (SPC) states that concomitant administration of CYP3A inducers and inhibitors should be avoided as they respectively reduce or increase the pharmacodynamic effects of irinotecan and SN-38 (Electronic Medicines Compendium, 2009).

It is therefore perhaps surprising that statistically significant correlations of CYP3A SNPs with irinotecan pharmacokinetics or toxicities have generally not been demonstrated (Mathijssen et al., 2003, Cote et al., 2007, Sai et al., 2008). This may be due to the low allele frequency of most CYP3A variant genotypes and the fact that this enzyme system is extensively influenced by external factors (co-medications, food substances) and internal factors (age, disease, hepatic and renal function) (Mathijssen et al., 2004).

Some potential in the use of CYP genotyping was demonstrated in the North American Gastrointestinal Intergroup Trial N9741, whereby the presence of the CYP3A5*3 (1334 T>C) variant was significantly associated with response rate in those receiving IFL (29% v 60%; P = 0.0074) (McLeod et al., 2010).
1.7.2.3.3 Uridine diphosphate glycosyltransferase (UGT)

Reduced expression of the UGT1A1 enzyme leads to lower glucuronidation rates and thus higher levels of, and/or prolonged exposure to SN-38 (Iyer et al., 2002). An increased number of TA repeats in the TATA box in the promoter region of this gene (wild type n=6) correlates with reduced enzyme expression (Iyer et al., 1999). UGT1A1*28 (defined by the presence of 7 TA repeats) is the most extensively investigated of the polymorphisms associated with irinotecan metabolism. Evidence for its association initially came from the observation that patients with Gilbert’s syndrome (the most common hereditary cause of elevated bilirubin, characterised by reduced expression of UGT1A1, most frequently due to UGT1A1*28) are susceptible to irinotecan toxicities (Wasserman et al., 1997). UGT1A1*28 has since been investigated in numerous studies outlined in Table 1-6.

In 2005, UGT1A1*28 kits (Hasegawa et al., 2004) became commercially available and the US Food and Drug Administration (FDA) Advisory Committee on Pharmaceutical Sciences recommended that patients homozygous for UGT1A1*28 receive a lower starting dose of irinotecan. This advice was made on the basis of 4 trials demonstrating the association between this genotype and irinotecan toxicities in a total of 30 patients homozygous for UGT1A1*28 (Innocenti et al., 2004, Marcuello et al., 2004, Rouits et al., 2004, Ando et al., 2000).

In 2007 a meta-analysis of 9 studies, including dosing regimens that were not initially reviewed by the FDA committee, concluded that the risk of haematological toxicities was increased with UGT1A1*28 only at medium or high doses of irinotecan (>150 mg/m²). There was no association with the risk of diarrhoea (Hoskins et al., 2007). In the FOCUS study (the largest CRC RCT to assess candidate pharmacogenetic markers to
date) there was no significant association of UGT1A1*28 with toxicity in patients receiving either irinotecan monotherapy or the FOLFIRI combination (Braun et al., 2009). Explanations for the conflicting results of these later studies include: the relatively small patient numbers used in some studies, the use of different irinotecan doses and combination regimens, the use of retrospective analysis and the confounding of data by environmental factors and other genetic variants (Toffoli et al., 2006, van der Bol et al., 2010). A further limitation is that the UGT1A1*28 allele frequency is higher in Caucasians than in Asians in whom UGT1A1*6 (211 G>A) is more commonly associated with Gilbert’s syndrome and thus may be predictive of toxicity (Han et al., 2006, Jada et al., 2007).

Whilst there is undoubtedly, a dose modulated association between UGT1A1*28 and the pharmacokinetics of SN-38, routine testing for this polymorphism to has not been adopted worldwide owing to the presence of conflicting negative data and thus lack of endorsement by specialist societies (Ross et al., 2010). It has been shown that the recommended dose of 180 mg/m² for irinotecan in the FOLFIRI regimen is considerably lower than the dose that can be tolerated when patients with the UGT1A1*28/*28 genotype are excluded, thus many patients may be being undertreated (Toffoli et al., 2009, Marcuello et al., 2011). It may be therefore be that this test could be used to optimise dose intensity in those at lower risk of toxicities.

There is also conflicting evidence on the effect of homozygosity for UGT1A1*28 predicting response with some studies demonstrating a trend towards higher response rates (Toffoli et al., 2006, Ando et al., 2000, Carlini et al., 2005) and others no difference (Marcuello et al., 2004, Liu et al., 2008, Kweekel et al., 2008b).
Table 1-6. The association of the UGT1A1*28 polymorphism with irinotecan toxicities.

<table>
<thead>
<tr>
<th>Study description &amp; treatment regimen</th>
<th>Genotype (number of TA repeats)</th>
<th>Patient number (%)</th>
<th>Number with grade III/IV neutropaenia (%)</th>
<th>Patient number with grade III/IV diarrhoea (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case control retrospective. Any irinotecan containing regimen &lt;60 to &gt;600 mg/m² weekly to once every 4 weeks</td>
<td>6/6 6/7 7/7</td>
<td>93 (79) 18 (15) 7 (6)</td>
<td>14 (15) † 8 (44)† 4 (57)†</td>
<td>(Ando et al., 2000)</td>
<td></td>
</tr>
<tr>
<td>Phase I prospective. Irinotecan 300 mg/m² every 3 weeks</td>
<td>6/6 6/7 7/7</td>
<td>9 (45) 7 (35) 4 (20)</td>
<td>0 (0) 0 (0) 2 (50)</td>
<td>0 (0) 1 (14) 1 (25)</td>
<td>(Iyer et al., 2002)</td>
</tr>
<tr>
<td>Phase I prospective. Irinotecan 350 mg/m² every 3 weeks</td>
<td>6/6 6/7 7/7 Other</td>
<td>30(46.2) 25(38.5) 6 (9.2) 4</td>
<td>0 (0) 3 (12) 3 (50)* NR</td>
<td>0 (0) 2 (8) 1 (17) NR</td>
<td>(Innocenti et al., 2004)</td>
</tr>
<tr>
<td>Prospective observational. Irinotecan monotherapy or combination regimens 80 – 350 mg/m² weekly to once every 4 weeks</td>
<td>6/6 6/7 7/7</td>
<td>40 (42) 45 (47) 10 (11)</td>
<td>6 (15) 12 (27) 4 (40)</td>
<td>7 (17) 15 (33)* 7 (70)*</td>
<td>(Marcuello et al., 2004)</td>
</tr>
<tr>
<td>Retrospective observational. Irinotecan and 5FU combination regimens (85 - 180 mg/m²) weekly to once every 4 weeks</td>
<td>6/6 6/7 7/7 Other</td>
<td>31(41) 35 (47) 7 (9) 2 (3)</td>
<td>3 (10) 14 (40) * 5 (71)*</td>
<td>4 (13) 7 (20) 2 (29)</td>
<td>(Rouits et al., 2004)</td>
</tr>
<tr>
<td>Phase II prospective. Cape/IRI 100 – 125 mg/m² weekly</td>
<td>6/6 6/7 7/7 Other</td>
<td>28 (42) 29(44) 5(8) 4(6)</td>
<td>10 (34) † 11(38) † 0 (0) † NR</td>
<td>(Carlini et al., 2005)</td>
<td></td>
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<tr>
<td>Phase II prospective. Raltitrexed and irinotecan 80 mg/m² weekly</td>
<td>6/6 6/7 7/7</td>
<td>27 (48) 22 (39) 10 (13)</td>
<td>1 (4) 2 (9) 1 (14)</td>
<td>4 (15) 8 (36) 1 (14)</td>
<td>(Massacesi et al., 2006)</td>
</tr>
<tr>
<td>Prospective observational. FOLFIRI biweekly (180mg/m² biweekly)</td>
<td>6/6 6/7 7/7</td>
<td>114 (46) 114 22 (9)</td>
<td>12 (11) 21 (18) 4 (18) ‡</td>
<td>18 (16) 20 (17) 5 (23) ‡ NR</td>
<td>(Toffoli et al., 2006)</td>
</tr>
<tr>
<td>Prospective phase II. Irinotecan and cisplatin 80 mg/m² every 3 weeks for NSCLC.</td>
<td>6/6 6/7 7/7</td>
<td>69 (85) 12 (15) 0 (0)</td>
<td>18 (26) 4 (33) 1 (0)</td>
<td>7 (10) 1 (8)</td>
<td>(Han et al., 2006)</td>
</tr>
<tr>
<td>Prospective phase III FOLFIRI biweekly (180 mg/m² biweekly)</td>
<td>6/6 6/7 7/7</td>
<td>79 (45) 81 (46) 16 (9)</td>
<td>5 (6) 10 (12) 4 (25)</td>
<td>6 (8) 12 (15) 2 (13)</td>
<td>(Cote et al., 2007)</td>
</tr>
<tr>
<td>Study description &amp; treatment regimen</td>
<td>Genotype (number of TA repeats)</td>
<td>Patient number (%)</td>
<td>Number with grade III/IV neutropenia (%)</td>
<td>Patient number with grade III/IV diarrhoea (%)</td>
<td>Reference</td>
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<tr>
<td>Prospective observational</td>
<td>6/6</td>
<td>59 (41)</td>
<td>9 (15)</td>
<td>NR</td>
<td>(Ruzzo et al., 2008)</td>
</tr>
<tr>
<td>FOLFIRI biweekly (180 mg/m² biweekly)</td>
<td>6/7</td>
<td>72 (49)</td>
<td>13 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>15 (10)</td>
<td>12 (80)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective observational</td>
<td>6/6</td>
<td>61 (48)</td>
<td>1 (2)</td>
<td>14 (23)</td>
<td>(Kweekel et al., 2008b)</td>
</tr>
<tr>
<td>Cape/IRI every 3 weeks (250 mg/m²)</td>
<td>6/7</td>
<td>58 (46)</td>
<td>4 (7)</td>
<td>14 (24)</td>
<td></td>
</tr>
<tr>
<td>or irinotecan monotherapy every 3</td>
<td>6/6</td>
<td>8 (6)</td>
<td>2 (25) *</td>
<td>4 (50)</td>
<td></td>
</tr>
<tr>
<td>weeks (350 mg/m²)</td>
<td>6/7</td>
<td>44 (57)</td>
<td>4 (2)</td>
<td></td>
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<tr>
<td></td>
<td>7/7</td>
<td>30 (39)</td>
<td>1 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrospective observational</td>
<td>6/6</td>
<td>102 (80)</td>
<td>5 (5)</td>
<td>6 (6)</td>
<td>(Liu et al., 2008)</td>
</tr>
<tr>
<td>FOLFIRI biweekly (180 mg/m² biweekly)</td>
<td>6/7</td>
<td>20 (15)</td>
<td>14 (54) * #</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>6 (5)</td>
<td>7 (27) #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective phase III</td>
<td>6/6</td>
<td>322 (52)</td>
<td>84 (26%)</td>
<td></td>
<td>(Braun et al., 2009)</td>
</tr>
<tr>
<td>S FU</td>
<td>6/7</td>
<td>225 (36)</td>
<td>50 (22%)</td>
<td></td>
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<tr>
<td></td>
<td>7/7</td>
<td>71 (11)</td>
<td>20 (28%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLFOX</td>
<td>6/6</td>
<td>132 (51)</td>
<td>75 (57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td>105 (41)</td>
<td>48 (46%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>22 (8)</td>
<td>13 (59%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLFIRI (180 mg/m² biweekly)</td>
<td>6/6</td>
<td>133 (55)</td>
<td>49 (37%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td>90 (37)</td>
<td>34 (38%)</td>
<td></td>
<td></td>
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<td></td>
<td>7/7</td>
<td>19 (8)</td>
<td>10 (53%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irinotecan (350 mg/m² every 3 weeks)</td>
<td>6/6</td>
<td>82 (48)</td>
<td>35 (43%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td>69 (40)</td>
<td>27 (39%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>20 (12)</td>
<td>10 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective phase III</td>
<td>6/6</td>
<td>44 (40)</td>
<td>3 (7)</td>
<td></td>
<td>(McLeod et al., 2010)</td>
</tr>
<tr>
<td>IFL (100 – 125 mg/m²) weekly for 4</td>
<td>6/6</td>
<td>54 (50)</td>
<td>6 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of 6 weeks</td>
<td>6/7</td>
<td>11 (10)</td>
<td>2 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>11 (10)</td>
<td>2 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLFOX</td>
<td>6/6</td>
<td>134 (47)</td>
<td>25 (19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td>126 (44)</td>
<td>28 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>27 (9)</td>
<td>10 (36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IROX (200 mg/m² every 3 weeks)</td>
<td>6/6</td>
<td>52 (50)</td>
<td>5 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td>40 (39)</td>
<td>6 (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7/7</td>
<td>11 (11)</td>
<td>6 (55)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Doses are only shown for irinotecan. For details of regimens refer to original reference.
FOLFOX is oxaliplatin combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
FOLFIRI is irinotecan combined with bolus and infusional 5-FU and leucovorin every 2 weeks.
IFL is irinotecan combined with bolus 5-FU and leucovorin weekly for 4 weeks every 6
IROX is irinotecan and oxaliplatin every 2 weeks
CapeIRI is irinotecan and capecitabine every 3 weeks
NR not reported, NSCLC none small cell lung cancer
* Significant for toxicity (p<0.005) compared to 6/6
† Data for grade III / IV diarrhoea and neutropaenia analysed together
‡ Significant for toxicity compared to 6/6 (p<0.005) for 1\textsuperscript{st} cycle of treatment only
∞ Diarrhoea and other non haematological toxicities
¥ Febrile neutropaenia only reported
# 6/7 and 7/7 analysed together
There are many other polymorphisms of the UGT families but only few of clinical relevance have been described so far. Data remain inconsistent, thus these potential biomarkers fail to be validated (Table 1-7). Data interpretation is complicated by the fact that many of these variants occur in the same gene cluster so linkage disequilibrium occurs. Each polymorphism may therefore not be independently contributing to the risk of toxicities. Examples include 75% of patients homozygous for UGT1A1*28 additionally exhibit UGT1A7 variants (Lankisch et al., 2005) and in Asian patients polymorphisms in UGT1A7 and UGT1A9 are closely linked to UGT1A1*6 (Han et al., 2006, Fujita et al., 2007). Multivariate analysis may therefore diminish an association apparent on univariate analysis (Kitagawa et al., 2005). Haplotype based analysis have therefore been conducted and some have demonstrated improved prediction of outcome. The presence of UGT1A1*28, *60, *93 and UGT1A7*3 predicts severe haematological toxicity in patients receiving FOLFIRI (OR, 0.39, p=0.01) (Cecchin et al., 2009).
Table 1-7. The UGT variants (excluding UGT1A1*28) that may be associated with irinotecan toxicities.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Description</th>
<th>Associated toxicities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1*6</td>
<td>211 G&gt;A</td>
<td>Increased grade 4 neutropaenia in AA homozygotes. Asian populations only.</td>
<td>(Han et al., 2006) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased grade 4 neutropaenia in those with at least 1 A allele. Asian populations only.</td>
<td>(Jada et al., 2007) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None.</td>
<td>(Ando et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None.</td>
<td>(Carlini et al., 2005)</td>
</tr>
<tr>
<td>UGT1A1*27</td>
<td>686 C&gt;A</td>
<td>All 3 patients heterozygous (CA) experienced either severe diarrhoea or neutropaenia.</td>
<td>(Ando et al., 2000)</td>
</tr>
<tr>
<td>UGT1A1*60</td>
<td>3279 T&gt;G</td>
<td>Increased grade 3/4 toxicities in GG homozygotes.</td>
<td>(Kitagawa et al., 2005) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased 1st cycle haematological toxicity in GG homozygotes.</td>
<td>(Cecchin et al., 2009) *</td>
</tr>
<tr>
<td>UGT1A1*93</td>
<td>3156 G&gt;A</td>
<td>Lower neutrophil nadir in AA homozygotes.</td>
<td>(Innocenti et al., 2004, Innocenti et al., 2009)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased grade 3/4 haematological toxicity AA homozygotes.</td>
<td>(Cote et al., 2007) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None.</td>
<td>(Cecchin et al., 2009)</td>
</tr>
<tr>
<td>UGT1A7*3</td>
<td>387 T&gt;G</td>
<td>Increased 1st cycle haematological toxicity in *3 homozygotes.</td>
<td>(Cecchin et al., 2009)*</td>
</tr>
<tr>
<td></td>
<td>391 C&gt;A</td>
<td>Increased severe diarrhoea in *3 homozygotes.</td>
<td>(Han et al., 2006)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased thrombocytopenia and diarrhoea in those with at least one *3 allele.</td>
<td>(Lankisch et al., 2008)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Reduced</strong> toxicity in *3 homozygotes.</td>
<td>(Carlini et al., 2005)*</td>
</tr>
<tr>
<td>UGT1A9 *22</td>
<td>T insertion / deletion at position – 118 T 9&gt;10</td>
<td><strong>Reduced</strong> risk of grade 3/4 toxicities in 9/9 homozygotes.</td>
<td>(Carlini et al., 2005)*</td>
</tr>
<tr>
<td></td>
<td>118 T 9&gt;10</td>
<td>Increased grade 3/4 diarrhoea in 9/9 homozygotes.</td>
<td>(Han et al., 2006) *</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Reduced</strong> risk of grade 3/4 neutropaenia in 9/9 homozygotes.</td>
<td>(Cecchin et al., 2009) *</td>
</tr>
</tbody>
</table>

* statistically significant studies with p<0.05

1.7.2.3.4 Membrane Transporters

Common polymorphisms in genes encoding for ABC and SLC transporters have a significant impact on the pharmacokinetics and pharmacodynamics of irinotecan (Sai et al., 2003, Mathijssen et al., 2003, Han et al., 2007, Fujita et al., 2008, Innocenti et al., 2009). Whilst the evidence is convincing that reduced expression of an ABC pump
reduces excretion of the drug and its metabolites, the evidence correlating the presence of these SNPs to toxicities and response is weak and frequently conflicting. SNPs including those at positions 2677 and 3435 in the ABCB1 gene lead to decreased clearance of SN-38 and have been associated with a higher incidence of grade 4 neutropenia (P =0.03) and diarrhoea (p=0.047) respectively (Han et al., 2007). Other studies however, failed to show a correlation of SNPs in ABCB1 with toxicity or dose reductions (Cote et al., 2007, Braun et al., 2008).

There is evidence that impaired ABCC2 activity, leading to lower hepatobiliary irinotecan clearance, is associated with a reduced incidence of diarrhoea (de Jong et al., 2007) but there are also conflicting data (Han et al., 2007).

Variants in the SLCO1B1 gene may be associated with toxicities. In patients with NSCLC, grade 4 neutropenia was significantly associated with the 521T>C SNP, whereas the 388A>G SNP was associated with grade 3 diarrhoea (Han et al., 2008).

1.7.2.3.5 DNA repair genes

Results from studies correlating DNA repair genotypes with toxicities have also been inconsistent. In the FOCUS study, a G>A SNP at position 23,885 in the base excision repair gene X-Ray Cross-Complementing Group 1 (XRCC1) was associated with toxicity induced dose delay / reduction in any irinotecan containing regimen. An A>C SNP at position 35,951 in the nucleotide excision repair gene Excision Repair Cross-Complementing gene 2 (ERCC2) was associated with increased toxicities in irinotecan monotherapy only (Braun et al., 2009). However, no association was noted in the North American Gastrointestinal Intergroup Trial N9741 (McLeod et al., 2010).
1.7.2.3.6 Other polymorphisms

The glutathione-S-transferase P1 (GSTP1) gene may protect from irinotecan induced apoptosis \textit{in vitro} (Goto et al., 2002). In the FOCUS study, an A>G SNP at position 313 in GSTP1 was weakly associated with toxicities to any irinotecan containing regimen (Braun et al., 2009).

1.7.3 Multivariate models

As so many SNPs have been associated with irinotecan effect, it may be that a panel of SNPs needs to be assessed. One SNP may influence the effect of another, for example, ABCC2 1019A>G only reduces the incidence of diarrhoea in UGT1A1 wild type individuals (de Jong et al., 2007).

Clinical determinants such as performance status and co-medication also influence irinotecan effect and should be taken into consideration when aiming and to develop predefined dosing algorithms (Kwekel et al., 2008a).

When a multivariate, genetic model was applied to the incidence of toxicities, 3 SNPs: ABCC1 IVS11 (8A>G), UGT1A1*93 and SLCO1B1*ib (388A>G), neutrophil baseline levels, sex and race explained almost half of the observed variation in absolute neutrophil count at nadir (Innocenti et al., 2009).

1.7.4 Summary statement predictive tests

A predictive test of irinotecan effect is highly sought. Although some modest associations with toxicities and response have been demonstrated, results of clinical studies have often been conflicting and an adequate clinically robust test that can change prescribing habits for the majority remains undiscovered. Genetic testing has shown the most potential, however at least half of the variation in toxicity and drug
exposure is unexplained by genotype (Innocenti et al., 2009). None of these techniques outlined have been widely adopted because evidence from adequately powered prospective clinical trials is still lacking. It is currently unclear whether any of these approaches will ultimately lead to individualised use of this drug. Further prospective clinical studies are required.

1.8 Developing a genotoxic predictive test of irinotecan toxicity in mCRC

1.8.1 Hypothesis – “DNA damage is a biomarker of irinotecan effect”

This hypothesis is based on the reports that the toxicities of irinotecan are due to the over-accumulation of SN-38 (section 1.6.5). As SN-38 causes DNA damage, measures of this damage on readily accessible normal cells, e.g. peripheral blood lymphocytes (PBLs) could therefore theoretically represent ideal biomarkers of its normal tissue toxicity. One may also expect that higher circulating SN-38 levels, and therefore higher DNA damage levels, would be associated with improved tumour response to treatment. However, this latter statement is controversial as data evidencing that those presumed to have high circulating SN-38 levels (i.e. UGT1A1*28 homozygotes) have improved response to treatment are inconsistent (section 1.7.2.3).

A weakness of the predictive tests of irinotecan effect that have been developed to date is that they fail to take into account all of the enzymes, transporters and environmental factors that are (known and unknown) to be involved in its complex metabolism. As DNA damage is the endpoint of its effect, one could speculate that it would therefore be a strong surrogate marker for of all of the factors affecting SN-38 metabolism and its binding to topo-I. Thus, if this hypothesis was proven to be true,
there would be a huge advantage in delivering a predictive test over those methods already researched (section 1.7.2).

1.8.2 Peripheral blood lymphocytes (PBLs) as a normal tissue surrogate

PBLs are frequently used in studies as a “surrogate normal tissue”. Their main advantages are that they: a) are readily available in large numbers with minimal risk and discomfort to the patients, b) circulate throughout the body, communicating with cells and extracellular matrixes in almost all tissues and organs, thus may be damaged in any target tissue by a toxic substance and c) contain genomic DNA so in theory their gene expression profiles should be representative of other normal tissues. The main disadvantage is that the biological effects observed in surrogate cells may not be the same in target cells.

Whether the study of PBLs may be used to predict tumour response to cytotoxic drugs is also controversial. It has long been reported that in vitro incubation of PBLs with antineoplastic drugs may predict the response to chemotherapy (Oshita et al., 1995). However there are also reports that PBL damage produced by combined chemotherapy does not reflect tumour response (Nadin et al., 2006).

The concept of using PBLs to assess inter-individual differences in metabolic pathways and thus predict toxicities is supported by evidence that 5-FU causes grade IV neutropenia at a higher frequency (55%) in those detected to have reduced DPD activity in PBLs compared to those with normal levels (13%) (van Kuilenburg et al., 2000).

There are data confirming that inter-individual differences in glucuronidation can be detected by studying PBLs both in vivo in a rat model and ex vivo in human samples. In a rat model the glucuronidation of the carcinogen benzo(a)pyrene (BP) in PBLs
accurately reflected the glucuronidation occurring in the livers from the same animals (Hu and Wells, 1994). The human study demonstrated that decreased glucuronidation of both BP diols and diones correlated with enhanced cytotoxicity (Hu and Wells, 2004). As glucuronidation is fundamental in the metabolism of irinotecan, these data provide further evidence to support the proposal to use PBLs as a surrogate to predict irinotecan effect.

There are also data demonstrating that amifostine protects PBLs cultured in the presence of a mitogen, from the DNA damaging effects of irinotecan as measured using sister chromatid exchange (Lialiaris et al., 2009). This provides further support to the hypothesis that DNA damage in PBLs may be a biomarker of irinotecan effect.

1.8.3 Methods used to measure DNA damage

Several methods may be used to measure DNA damage, each with their own specific advantages and drawbacks. Methods that were traditionally used (e.g. the alkaline elution assay) were not very sensitive and technically cumbersome (Tice et al., 2000). For that reason, more recently developed techniques namely the alkaline comet assay (ACA) and detection of γ-H2AX are used in this study. More detailed accounts of these techniques are given in sections 3.5 and 3.6.

1.8.3.1 Alkaline Comet Assay

The comet assay (single-cell gel electrophoresis assay) was initially described in 1984 by Östling and Johanson whose protocol performed under neutral conditions (pH 7-8) reportedly detected DSBs. In 1988, Singh et al then developed the assay to be performed under alkaline conditions (pH>13) (Singh et al., 1988). The alkaline pH ensured that SSBs, alkali labile sites (ALS) and incomplete excision repair sites could be
detected in addition to the DSBs. The alkaline version of this assay is far more sensitive at detecting DNA damage induced by genotoxic agents because the majority of these induce SSBs and / or ALS in a greater order of magnitude than they do DSBs (Tice et al., 2000).

The ACA and its applications has been widely reviewed elsewhere (Tice et al., 2000) (Brendler-Schwaab et al., 2005, Burlinson et al., 2007, Collins et al., 2008, Dusinska and Collins, 2008, Cavallo et al., 2009). The main advantages of the ACA are that it is: a) sensitive to detect low levels of DNA damage at pharmacologically relevant doses, b) relatively cheap, c) requires only a small number of cells, d) can be performed on any eukaryotic cell type, e) detects several types of DNA damage and f) yields results within only a few days.

The ACA is used in a wide range of applications including human and environmental biomonitoring, genotoxicity and DNA repair studies. There is evidence that the ACA has the potential to predict treatment effect; when irradiating 6 bladder cancer cell lines with differing radiosensitivities the extent of comet formation strongly correlated with cell killing (Moneef et al., 2003).

1.8.3.1.1 Using the ACA to measure DNA damage in PBLs in cancer patients

PBLs from healthy volunteers and cancer patients have been widely studied using the ACA. Many in vivo studies have shown that cancer patients have higher levels of basal damage than healthy persons with inter-individual variations between them (Blasiak et al., 2004, Nadin et al., 2006, Nadin et al., 2007, Sanchez-Suarez et al., 2008) although conflicting data demonstrate no difference between background DNA damage levels in cancer sufferers and healthy volunteers (Rajaee-Behbahani et al., 2001).
DNA damage induced by chemotherapeutic drugs on PBLs has also been assessed using variations of the ACA. A biomonitoring study reported that increased DNA damage may be detected in nurses due to occupational exposure to anti-neoplastic drugs (Rekhadevi et al., 2007). *In vivo* studies on cancer patients demonstrate that DNA damage is detectable in response to several drugs including: cyclophosphamide (Vaghef et al., 1997), doxorubicin (Blasiak et al., 2004, Nadin et al., 2006), cisplatin (Nadin et al., 2006) and poly-chemotherapy (Kopjar et al., 2002). Investigators have also successfully detected DNA damage following *ex vivo* exposure to paclitaxel (Branham et al., 2004), cisplatin and doxorubicin (Nadin et al., 2006).

Repair studies have shown that cancer patients have decreased repair efficacy, compared to healthy volunteers, towards oxidative and alklyative DNA damage (Blasiak et al., 2004) and to cisplatin (Nadin et al., 2006). The repair of doxorubicin induced damage however showed no significant difference between between cancer sufferers and healthy volunteers (Nadin et al., 2006).

Only a few of these chemotherapy studies have correlated the laboratory results with the clinical response and the results have been mixed. DNA damage levels induced with cisplatin exposure *ex vivo* demonstrated potential in correlating with tumour response however there was no association between DNA damage and clinical response for doxorubicin (Nadin et al., 2006) or adjuvant combination chemotherapy for breast cancer (Sanchez-Suarez et al., 2008). Systemic DNA damage appeared to be related to levels of the active metabolites of doxorubicin and ifosfamide but this did not correlate with response or toxicities (Johnstone et al., 2000).

ACA analysis of PBLs obtained from patients undergoing treatment in a phase I study demonstrated that DNA damage induced by temozolomide can be prolonged by co-
administration of a poly(ADP-ribose) polymerase (PARP) inhibitor (Plummer et al., 2008).

The ACA has not been widely used to assess irinotecan induced DNA damage in PBLs. One small study (n=20) demonstrated that there were increased DNA damage levels in PBLs of cancer sufferers following an ex vivo exposure to irinotecan than those from healthy volunteers. In addition, vitamins A, C and E further increased the amount of DNA damage (Kontek et al., 2009). Prior to embarking on this clinical study, preliminary data to support the research proposal were demonstrated in our laboratory. DNA damage induced by irinotecan exposure in vivo in PBLs was measured. Results indicated that increased levels of damage 1 hour following treatment correlated with patient toxicity (Smith et al., 2007). The increase in percentage tail DNA in this study however was only slight (2-4%) and patient numbers were very small (n=4). However, the strong scientific rationale (summarised above) and this promising early data (Smith et al., 2007) provided sufficient evidence to warrant expanding this research into a prospective clinical study.

1.8.3.2  Measuring phosphorylated H2AX

H2AX is a member of the histone H2A family. Its role is to recruit DNA repair and cell-cycle checkpoint proteins required for processing DNA DSBs. During the 30 minutes after DSB formation; 3 kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia Rad3-related (ATR) and DNA-depandent protein kinase (DNA-PK) lead to the phosphorylation of the serine 139 on the H2AX to form γ-H2AX (Rogakou et al., 1999). Proteins involved in DNA repair e.g. MRE11-RAD50-NBS1 (MRN) complex, RNF8, BRACA1 and p53 binding protein accumulate at γ-H2AX foci.
In the laboratory, γ-H2AX foci represent the concentration of repair proteins in the vicinity of DNA damage and may therefore be measured as a surrogate for DSB detection (reviewed in (Bonner et al., 2008, Rothkamm and Horn, 2009). The majority of DSBs form a γ-H2AX focus with only a few exceptions e.g. DNA ends in the topoisomerase II-DNA complex and the double stranded DNA end in telomeres do not form γ-H2AX. Although it is accepted that the majority if DSBs form a γ-H2AX focus, whether every γ-H2AX focus identifies a DSB remains controversial, however this method is widely regarded as being the most sensitive way to detect DSBs (Bonner et al., 2008).

Immunocytochemical methods using antibodies raised to the phosphorylated serine 139 terminal are used to detect γ-H2AX foci. Foci may be measured by either immunofluorescence microscopy or by flow cytometry. Immunofluorescence microscopy is the most sensitive method as each distinct foci most likely represents a single DSB. The main disadvantage of this method is that it is highly labour intensive. On the other hand FACS analysis measures the total fluorescence intensity for each cell and thus allows rapid detection of γ-H2AX in a large number of cells but is less sensitive to detect low levels of damage compared to the foci method (Ismail et al., 2007, Rothkamm and Horn, 2009).

Studies of blood cells demonstrate that γ-H2AX foci may be a useful quantitative biomarker of human diagnostic and therapeutic radiation exposure in vivo (Rothkamm et al., 2007, Sak et al., 2007). Cytotoxic drug induced damage has also been detected in vivo in peripheral blood mononuclear cells (PBMCs) from patients treated for acute leukaemia. Those who received clofarabine and cyclophosphamide had greater γ-H2AX levels compared with those who received cyclophosphamide alone (Karp et al., 2007).
Ex vivo studies have shown that damage induced to PBMCs with ionizing radiation and the DSB-inducing drug calicheamicin can be detected and there is a 2 fold inter-individual difference in γ-H2AX foci between individuals (Ismail et al., 2007) 

The predictive value of the γ-H2AX assay has also been assessed. Measurements of γ-H2AX levels in tumour tissues may predict response to treatment, e.g. the persistence of γ-H2AX foci is a marker of tumour sensitivity to radiation damage (Klokov et al., 2006). γ-H2AX formation also correlates with DNA damage associated with interstrand crosslinking (ICL) agents (Clingen et al., 2008).

1.9 Outline of prospective clinical study

The data presented in this introduction clearly demonstrate that the development of a predictive test of irinotecan effect could substantially improve the treatment for patients with metastatic CRC. Individualised dosing of this drug would have the potential to a) minimise toxicities, thereby improve quality of life, and b) maximise response, thereby improve survival for these patients. The extensive research detailed in this chapter, conducted to date, has failed to develop such a test to reliably predict irinotecan effect and thus improve routine clinical practice. This current study was therefore designed with the aim of developing a superior method to predict toxicities and response to irinotecan chemotherapy.

Presented in this thesis is a novel, prospective clinical study, assessing whether DNA damage induced in PBLs is a biomarker of irinotecan effect. Preliminary data, prior to commencing this work, had purported that in vivo irinotecan induced DNA damage in PBLs may correlate with its toxic effects (Smith et al., 2007). Clearly, in order to
develop a predictive test, it would be important to induce and detect DNA damage *ex vivo* before patients are exposed to the potentially toxic effects of the drug.

PBLs were therefore obtained from patients before and after receiving irinotecan chemotherapy and the DNA damage induced following *in vivo* drug exposure was investigated. In addition, DNA damage induced *ex vivo* was studied by exposing the PBLs obtained prior to administration of irinotecan chemotherapy to irinotecan or SN-38 in the laboratory. DNA damage was assessed by using the ACA and by measuring γ-H2AX. Polymorphisms known to be associated with slow deactivation of SN-38, and thus toxicities to irinotecan treatment, were also investigated. Associations between DNA damage levels and a) toxicities to treatment, b) clinical response to treatment and c) the presence or absence of these polymorphisms were then sought in order to determine whether DNA damage is a biomarker of irinotecan effect with the potential to individualise its use in the clinic.
2 Aims

1) To determine whether irinotecan treatment induces DNA damage in PBLs in vivo that can be measured using the alkaline comet assay.

2) To ascertain whether inter-individual differences in these in vivo DNA damage levels exist and if so whether these levels correlate with toxicities and response to treatment in patients receiving irinotecan chemotherapy.

3) To optimise a method to induce DNA damage ex vivo using irinotecan or its active metabolite SN-38, that can then be measured using the ACA and by detection of γ-H2AX.

4) To ascertain whether inter-individual differences in these ex vivo DNA damage levels exist and if so, to establish in clinical samples whether these levels correlate with toxicities and response to irinotecan chemotherapy.

5) To study the clinical samples in order to detect polymorphisms reported to have significant associations with irinotecan toxicity, namely UGT1A1*28 and UGT1A1*93

6) To determine whether the UGT1A1*28 and UGT1A1*93 polymorphisms correlate with levels of induced DNA damage from both the in vivo and ex vivo experiments and with the toxicity and response data.
3 Materials and Methods

All chemicals and cell culture reagents were obtained from Sigma-Aldrich Company Limited unless otherwise stated.

3.1 Laboratory materials

3.1.1 Chemicals

- Acetonitrile HPLC grade (Fisher Scientific)
- Alexa Fluor™ goat anti-mouse IgG (Invitrogen)
- Annexin V kit (Bender MedSystems)
- Anti-phosphohistone H2AX mouse monoclonal antibody (Millipore)
- Big dye terminator ready reaction mix and big dye terminator buffer (Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester)
- Blood and cell culture DNA kit (Qiagen)
- DNA ladder (50 Base pair) (New England BioLabs)
- Ethanol (Fisher Scientific)
- Exonuclease I (New England BioLabs)
- Fetal calf serum (Invitrogen)
- Ficoll-Paque™ PLUS (GE Healthcare)
- GlutaMAX™ (Invitrogen)
- Primers (Biomers.net)
- Quantum 724 complete media for primary lymphocyte culture (PAA laboratories)
- Shrimp alkaline phosphatase (Fermentas)
- Trypsin/EDTA (USB Corporation)


- TBE buffer 10 X stock (Geneflow)

3.1.2 Suppliers’ addresses

- Abgene, Epsom, UK
- Agilent Technologies, Stockport, UK
- Andor Technology, Belfast, Northern Ireland
- Applied Biosystems, Warrington, UK
- Beckman Coulter, High Wycombe, UK
- Becton Dickenson, Oxford, UK
- Bender MedSystems, Vienna, Austria
- Biomers.net, Ulm, Germany
- Carl Zeiss Ltd, Welwyn Garden City, UK
- Corning Life Sciences, Pittsburgh, USA
- EdgeBio, Gaithersburg, USA
- Fermentas, York, UK
- Fisher Scientific, Loughborough, UK
- GE Healthcare, Buckingham, UK
- Geneflow, Staffordshire, UK
- Invitrogen, Paisley, UK
- LGC Promochem, Teddington, UK
- Millipore, Billerica, USA
- MJ Research, Waltham, USA
- NanoDrop Technologies, Delaware, USA
- New England BioLabs (NEB), Hitchin, UK
3.1.3 Buffers and media

- **Blocking buffer.** KCM washing buffer (see below) plus 2% bovine serum albumin (BSA), 10% milk powder and 10% normal goat serum, made immediately before use.

- **Buffer 3.** 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.9 stored at -20°C.
• **Cell lysis buffer C1.** 1.28 M sucrose, 40 mM Tris-Cl, 20 mM MgCl₂, 4% Triton X-100, pH 7.5 stored at 4°C.

• **Digestion buffer G2.** 800 mM guanidine HCl, 30 mM Tris-Cl, 30 mM EDTA, 5% Tween-20, 0.5% Triton X-100, pH 8.0 stored at 4°C.

• **Electrolysis buffer.** 300 mM NaOH, 1 mM disodium ethylenediaminetetraacetic acid (EDTA), pH 13. Prepared immediately prior to use from stock solutions of 10 M NaOH and 200 mM disodium EDTA with ice cold double distilled water (ddH₂O). Stock solutions were stored at room temperature.

• **Elution buffer QF.** 1.25 M NaCl, 50 mM Tris-Cl, 15% isopropanol, pH 8.5 stored at 4°C.

• **Equilibration buffer QBT.** 750 mM NaCl, 50 mM 3-(N-Morpholino) propane sulfonic acid (MOPS), 15% isopropanol, 0.15% Triton X-100, pH 7.0, stored at 4°C.

• **KCM washing buffer.** 120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA stored at 4 °C plus 0.1% triton X-100 added immediately before use.

• **Lysis buffer.** 100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH to 10.0 with 10 M NaOH stored at 4 °C plus 1% triton-X-100 added immediately prior to use.

• **Neutralisation buffer.** 0.4 M Tris-HCl, pH 7.5, stored at room temperature.

• **PBST.** PBS with 4% foetal calf serum (FCS) and 0.1% triton X-100 made immediately prior to use.

• **Tris/Borate/EDTA (TBE).** The 10 x stock was 890 mM Tris, 890 mM Boric acid, 20 mM EDTA, stored at room temperature.
- **Tris/EDTA.** The 50 x stock was 500 mM Tris, 50 mM EDTA, pH 7.5, stored at room temperature.

- **Trypsin/EDTA (TE).** 0.05% trypsin, 0.7 mM EDTA diluted in PBS stored at 4 °C

- **Wash buffer QC.** 1 M NaCl, 50 mM MOPS, 15% isopropanol, pH 7.0, stored at 4°C.

- **11.1 x PCR buffer:** 499.5 mM Tris-HCl [pH 8.8], 122.1 mM ammonium sulphate, 49.59 mM magnesium chloride, 0.5% 2-mercaptoethanol, 48.84 μM EDTA [pH 8.0], equal volumes (11.1 mM) of all 4 deoxynucleotide triphosphates (dNTPs), plus 144.3 μg/ml BSA stored at -20°C. Addition of 0.9μl of 11.1 x buffer gave the following final concentrations in a 10μl total volume PCR reaction mix: 45 mM Tris-HCl, 11 mM ammonium sulphate, 4.5 mM magnesium chloride, 0.045% 2-mercaptoethanol, 4.4 μM EDTA, 1 mM of each dNTP, and 13 μg/ml BSA.

3.1.4 Drug treatments

- **Irinotecan.** 80.24 mM solution was made by dissolving 50 mg of irinotecan powder in 1 ml of dimethylsulfoxide (DMSO) according to the manufacturer’s instructions.

- **Saquinavir.** 767.05 mM stock solution was prepared by dissolving 10 mg of saquinavir powder in 10 ml RPMI media with 0.1% DMSO.

- **SN-38.** 2.55 mM solution was prepared by dissolving 10 mg of SN-38 powder in 10 ml of DMSO according to the manufacturer’s instructions.

These drug stock solutions were stored at -20°C in 50 - 100 μl aliquots for up to one year. Aliquots were thawed prior to use and any unused drug was discarded. Serial dilutions, in the appropriate culture media for the cell line being treated, were used to
prepare the working concentration of drug. The final DMSO concentration was adjusted so that it was the same across all doses including the untreated control. Treatments were prepared immediately prior to dosing and were pre-warmed to 37°C in a water bath. To treat adherent cell lines, the old culture media was removed and the working concentration of drug was applied directly to the cells. To treat PBLs (i.e. suspension cells), 2 x working concentration of drug was added to an equal volume of cells suspended in media.

3.2 Established adherent cell lines

3.2.1 Cell culture materials

One colorectal adenocarcinoma cell line (HT29) and two Chinese hamster ovary (CHO) cell lines (AA8 and irs1SF) were used in this research. The HT29 cells (KRAS wild type, DNA mismatch repair proficient) were obtained from LGC Promochem. The CHO cell lines were kindly donated by Jennifer Anderson at the Gray Institute for Radiation Oncology and Biology in Oxford. Cell lines tested negative for mycoplasma infection. Cell culture was undertaken in a class II laminar flow hood.

3.2.2 Maintenance of cell lines

Cells were grown as a monolayer in 25 cm² culture flasks (Corning Life Sciences) in an incubator at 37°C with a humidified atmosphere of 5% CO₂ in air. The media used for each cell line was as follows:-

- **HT29.** Dubecco’s modified eagle’s medium (DMEM) with 4500 mg glucose/L, 110 mg sodium pyruvate/L and L-glutamine plus 10% FCS.
- **irs1SF.** DMEM with 1000 mg glucose/L, L-glutamine, NaHCO<sub>3</sub> and pyridoxine HCl plus 10% FCS.
- **AA8.** Minimum essential medium eagle (MEM) with alpha modification and NaHCO<sub>3</sub> without ribonucleosides and deoxyribonucleosides plus 10% FCS and 2% glutaMAX.

For long term storage in liquid nitrogen, cells were stored cryovials (SARSTEDT) containing 1 ml of freezing media. Freezing media consisted of the appropriate media for each cell line plus 20% FCS and 10% DMSO. Cells were slowly frozen for 24 hours at -20°C then for 24 hours at -80°C prior to being placed in the liquid nitrogen.

### 3.2.3 Passaging of cells

Cells were split twice weekly when approximately 60 – 70% confluent. A maximum of 35 passages were performed per cell line following which further cells were resurrected from liquid nitrogen. Cells were not used in experiments for at least 2 weeks following storage in liquid nitrogen to ensure that normal cell division had been restored.

To subculture the cells, 1 X TE, PBS and media were all pre-warmed to 37°C in a water bath. A separate bottle of media, PBS and TE was allocated to each cell line in order to avoid cross contamination. The old media was removed and cells were washed with 10 ml of PBS. Next 1ml of 1 X TE was added to the flask and left for approximately 5 minutes at 37°C in an incubator to allow detachment of the cells. Finally, 9 ml of media was added and this resulting cell suspension seeded into culture flasks at 1 in 10 and 1 in 100 dilutions for further culture (the 1 in 100 dilution was kept as a backup in case of contamination during subsequent subculture of the 1 in 10 cells).
3.2.4 Trypan blue exclusion viability assay

Cells were counted and viability assessed by mixing 20 μl of cell suspension with 20 μl of trypan blue then viewing on a haemocytometer. Both viable (non-stained) and non-viable (stained blue) cells were counted.

Cell number per ml of cell suspension was calculated using the formula:

\[
\text{Number of cells/ml} = \left(\frac{\text{number cells counted}}{\text{number grids counted}}\right) \times 2 \times 10^4
\]

\(10^4\) is the conversion of cells/0.1 mm\(^3\) (volume of grid) and 2 is the dilution factor.

3.2.5 Treatment of adherent cell lines with irinotecan or SN-38

The cell suspension produced following trypsinisation (section 3.2.3) was transferred to a universal container and the cells pelleted by centrifugation at 1500 rpm for 5 minutes at room temperature in an Allegra 6KR centrifuge (Beckman Coulter). The supernatant was discarded and the cells were resuspended in 10 ml of media and then counted using the trypan blue exclusion assay (section 3.2.4). Cells were seeded in 6-well plates in a final volume of 2ml of media at a density of either: a) 200,000 cells per well and left overnight to attach or b) 50,000 cells per well and left for 72 hours to attach prior to treatment. Following attachment, media was removed and 2 ml of the drug containing media applied (section 3.1.4). The treated cells were left for a specified time in the incubator at 37°C with 5% carbon dioxide following which the treatment media was removed; each well was washed with 2 ml of PBS and incubated for 5 minutes with 500 μl of 1 X TE to allow detachment of cells. 2 ml of media was then added and the cell suspension was transferred to a universal container and the cells pelleted by centrifugation at 1500 rpm for 5 minutes at 4°C. The cells were either used for an experiment immediately or the pellet was resuspended in 1ml of freezing

1 Unless otherwise stated centrifugation was always performed in the Allegra 6KR centrifuge (Beckman Coulter)
media (section 3.2.2) and slowly frozen prior to being stored at -80°C until the ACA was performed as detailed in section 3.5.

3.2.6 Irradiation of HT29 cells

The cell pellet formed following centrifugation of trypsinised cells (section 3.2.3) was resuspended in 1ml of ice cold PBS. Cells were counted using the trypan blue exclusion assay (section 3.2.4) and 90,000 cells were placed into individual ependorfs. They were then transported and irradiated on ice at a dose of 10 Gy (dose rate of 1 Gy / min; 250 kV constant potential, 1.2 mm Cu; Pantak industrial X-ray machine). These cells were slowly frozen in 750 µl of freezing medium (section 3.2.2) until the ACA was performed (section 3.5)

3.3 Peripheral blood lymphocyte (PBL) samples

3.3.1 Blood collection

Verbal consent was taken from healthy volunteers and written informed consent was obtained from each clinical patient prior to performing venepuncture (section 3.10.1). Unless otherwise stated, blood samples (10 – 20 ml) were collected in heparinised vials (SARSTEDT, Germany). Samples were coded and kept at room temperature until the PBLs were isolated as quickly as possible.

3.3.2 PBL extraction

All blood samples were processed in a class I hood. PBLs were isolated using density centrifugation with Ficoll-paque™ PLUS according to manufacturer’s instructions with minor modifications. Firstly, the blood was diluted with an equal volume of RPMI 1640 media that had been pre-warmed to 37°C in a water bath. 4 ml of this blood/RPMI
mixture was carefully layered on top of 3 ml Ficoll-paque™ PLUS in 15 ml falcon tubes (Corning) and centrifuged at 1700 rpm for 30 minutes at room temperature without braking. The interphase layer was collected with a Pasteur pipette and washed twice in RPMI 1640 media. Cells were counted and assayed for viability using the trypan blue exclusion assay (section 3.2.4). The isolated PBLs cells were either placed directly into culture media, or slowly frozen in freezing media (section 3.2.2) for 24 hours at -20°C prior to being stored at -80°C for use in future experiments. They were stored for up to one year. Frozen PBLs were only used to perform the ACA or for DNA extraction, whereas, fresh PBLs were used for further subculture and drug treatments.

3.3.3 Culture of PBLs
Following isolation, unless otherwise stated PBLs were resuspended in Quantum 724 complete media for primary lymphocyte culture (QBL). They were seeded at a density of 2.5 - 5 x 10⁵ /ml in 25 cm² flasks and cultured for 72 hours prior to being used for experiments. The flasks were gently agitated every 24 hours.

3.3.4 Treatment of PBLs with irinotecan or SN-38
Following culture for 72 hours, cells were counted and viability assessed using the trypan blue exclusion assay (section 3.2.4). Unless otherwise stated, PBLs were treated in 6-well plates. A 1 ml aliquot of lymphocyte suspension was pipetted into each well and then treated with 1 ml QBL media containing 2 x working solution of drug (section 3.1.4). Cells were treated for a predetermined time following which the contents of the well were transferred into universal containers and centrifuged at 1500 rpm for 5 minutes at 4°C. These cells were then processed in the various assays as described below.
3.4 Cell cycle analysis by flow cytometry

3.4.1 Principles of flow cytometry

Flow cytometry is a widely used system for measuring the signals that result from the flow of particles in a liquid stream through a beam of light (Givan, 2001). If the particles being analysed are stained with a fluorescent molecule, the colour and intensity of the emitted light can also be detected. The resulting information can then be analysed using appropriate computer software.

When performing cell cycle analysis, propidium iodide (PI) which only fluoresces when bound to double stranded DNA, or RNA, is added to the samples. Flow cytometry may then be used to assess a cell population for its DNA content; cells in the G₀ and G₁ phases of the cell cycle have the normal diploid DNA content (2n), cells in G₂ and M possess tetraploid DNA (4n) and cells in the S phase have varying amounts of DNA between 2 and 4n (Givan, 2001).

3.4.2 Cell cycle analysis method

The cell pellets formed following treatment and then removal of the drug containing media were resuspended in 200 µl of cold PBS and transferred to a 15 ml falcon tube. Cells were then fixed in 2 ml of ice cold 70% ethanol that was slowly added whilst the tube was held on a Genie 2 vortex (Scientific Industry) to prevent cell clumping. The ethanol was necessary to permeabilise the cell membranes in order for the stain to reach the nuclei. These samples were kept at -20°C (and could be kept like this for up to 4 weeks) until staining and analysis was performed.

To complete the staining process the samples were initially centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended
in 800 μl PBS and 100 μl RNase A prior to incubation in a waterbath at 37°C for 10 minutes. The RNase ensured that the detected fluorescence only resulted from the DNA and not RNA. 100 μl PBS containing 50 µg/ml PI was then added and samples were kept at 4°C overnight prior to analysis. Cell cycle distribution was determined using a Becton Dickinson fluorescence activated cell sorter (FACS). The resulting information was analysed using CellQuest software (Becton Dickinson, San Jose, CA) as illustrated in Figure 3-1.
Figure 3-1. Example of the processes involved in cell selection for cell cycle analysis distribution by flow cytometry.

A) Images were gated to exclude cell debris according to the side scatter (SSC) and forward scatter (FSC) parameters.
B) A second gate was then applied to exclude aggregates according to fluorescence peak width (FL2-W) and area (FL2-A). The nuclei fluoresced red (given by the FL2-A parameter) with an intensity proportional to the cells’ DNA content (Givan, 2001).
C) The number of cells per fluorescence intensity was then used to plot a DNA histogram of these gated cells. 1 x 10⁴ cells were analysed per sample.
D) Data analysis to estimate the percentage of cells in each phase was performed using ModFit LT software (Becton Dickinson). This software used mathematical algorithms, to determine the proportion of cells in each phase of the cell cycle.
3.5 The alkaline comet assay (ACA)

The ACA was the prime method employed to detect DNA damage during this study.

3.5.1 Principles of the ACA

The ACA was developed by Singh et al in the late 1980s (Singh et al., 1988) (section 1.8.3.1). The principles and general steps behind performing this assay are outlined below (Zainol et al., 2009):

1. **Slide preparation** with agarose gels. A single cell suspension is mixed with low melting point (LMP) agarose and placed on a microscope slide pre-coated with normal melting point (NMP) agarose. It is essential to pre-coat the slide to ensure that the gels adhere. The cells are mixed with LMP agarose at 37°C to ensure they are not damaged by excess heat. A coverslip is used to flatten out each molten agarose layer.

2. **Cell lysis** in the presence of high salt concentration and detergents liberates the DNA; the membranes and proteins (including histones) are removed. The remaining loops of negatively charged, supercoiled DNA that have lost most of the attached proteins (except some scaffolding proteins) are called the nucleoid body.

3. **Exposing the liberated DNA to alkali** disrupts the hydrogen bonds and allows the DNA to unwind. It also transforms alkali labile sites (ALS) to SSBs.

4. **Electrophoresing the DNA using pH > 13** results in nucleoid DNA being attracted to the anode. Only those loops containing a break, which relaxes the supercoiling, are free to unwind and migrate in the direction of electrophoresis to form a “tail” the undamaged DNA remains in the “head” (Figure 3-2)
5. **Alkali neutralization** is essential to enable the DNA to take up a stain.

6. **DNA staining** is achieved by using a dye e.g. propidium iodide (PI).

7. **Comet visualization** is performed using a fluorescence microscope.

8. **Analysis** can be performed manually, however, the use of purpose designed image analysis software is preferred. Several measures can be used to quantify the DNA damage (e.g. tail length, tail moment) but the most commonly described parameter is percentage tail DNA which is the most linearly related to dose (Kumaravel and Jha, 2006) and is calculated as follows:

\[
\text{Percentage tail DNA} = \frac{\text{Intensity of tail DNA}}{\text{Intensity of cell DNA}} \times 100
\]

**Figure 3-2. Comet visualisation using fluorescence microscopy**

**3.5.2 ACA method**

The following method was employed.

**3.5.2.1 Preparation of cells**

The cell pellets formed following treatment either *in vitro* (section 3.3.4) or *in vivo* (section 3.10.3) were resuspended in a volume of 250 µl; adherent cell lines were
resuspended in ice cold PBS whereas PBLs were resuspended in 0.9% saline. If cells had been frozen prior to performing the ACA then they were rapidly thawed in a water bath at 37°C, pelleted by centrifugation at 1500 rpm for 5 minutes at 4°C and the freezing media discarded prior to being resuspended as above. Cells were counted and viability assessed using the trypan blue exclusion assay. Appropriate volumes of cell suspension were transferred to pre-labelled eppendorf tubes to give approximately 2 x 10^4 cells per tube. These samples were then centrifuged at 300 x g in a Heraeus Fresco 21 centrifuge (Thermo Electron Corporation) for 5 minutes at 4°C and the supernatant discarded.

3.5.2.2 Making of gels
Gels were made using 0.6% low melting point (LMP) agarose in PBS that had been dissolved in a microwave and equilibrated to 37°C in a water bath; 170 µl of LMP agarose was added to the eppendorfs to resuspend the 2 x 10^4 cells and then 80 µl dispensed, in duplicate, onto glass microscope slides that had been pre-coated in 1% normal melting point (NMP) agarose in water, and held on an aluminium tray on ice. To minimise error, each treatment / condition was processed in triplicate within each individual experiment (2 gels per slide, 3 slides per treatment). Gels were allowed a minimum of 2 minutes to set on ice under a coverslip. Once set, the coverslips were removed and slides transferred to 25 ml Coplin jars containing ice cold lysis buffer (section 3.1.3). These jars were then packed on ice and kept at 4°C overnight.

3.5.2.3 Electrophoresis
Slides were removed from lysis buffer, placed gel side up in trays and washed twice with ice cold ddH₂O for 10 minutes taking care not to dislodge the gels. Next, they were transferred to an electrophoresis tank ensuring all slides were pointing the same
direction and that the electrophoresis tank was level and surrounded by ice. Triplicate slides from each sample were distributed in different parts of the tank. They were immersed in electrophoresis buffer (section 3.1.3), for 20 minutes. Electrophoresis was carried out in the same buffer for 20 minutes at 30 V (0.66 V / cm) and 300 mA and then the slides were removed from the tank, placed in trays and flooded with approximately 1 ml of neutralisation buffer for 20 minutes. They were washed twice in ddH₂O for 10 minutes before being transferred to the 37 °C drying oven until staining was performed.

### 3.5.2.4 Staining

Slides were rehydrated in ddH₂O for 30 minutes. The ddH₂O was removed and slides flooded with approximately 1 ml of 2.5 µl/ml freshly prepared PI solution for 20 minutes. They were rinsed and left in ddH₂O for a further 30 minutes and then were allowed to dry and stored in the dark until analysis.

### 3.5.2.5 Imaging

Gels were rehydrated with a drop of ddH₂O, following which a coverslip was placed over the gel, and the comets were visualised using a fluorescence microscope (Olympus) fitted with a 100 W Mercury Bulb at 200x magnification. Komet analysis software version 5.5 (Andor Technology) was used to capture and analyse the images. 50 comets were analysed in the central region of each gel. A total of 300 cells were therefore analysed per sample (50 per each of two gels on triplicate slides). In the event of a gel being lost during the procedure, 100 cells from the single gel on the same slide were scored. In the rare event of both gels from a slide being lost another of the triplicate slides was scored twice.
3.5.2.6 Analysis

STATGRAPHICS Centurion software (STATPOINT technologies) was used to remove significant outliers ($p < 0.05$). The median percentage tail DNA from each slide was calculated and unless otherwise stated the data presented are the mean values and the standard deviations of the median percentage tail DNA from these triplicates.

3.6 Measuring $\gamma$-H2AX

The second method for measuring DNA damage employed during the course of this study was DSB detection by measuring $\gamma$-H2AX levels.

3.6.1 Principles of measuring $\gamma$-H2AX

The H2AX histones usually become phosphorylated in response to DSBs, resulting in the formation of $\gamma$-H2AX foci, which may subsequently be detected by using antibodies raised to the phosphorylated terminal. Two immunocytochemical methods were used to detect $\gamma$-H2AX, the first of which relied on fluorescence microscopy to visualise and count foci directly and the second method used flow cytometry to quantify $\gamma$-H2AX levels by measuring the total fluorescence intensity for each cell (section 1.8.3.2).

3.6.2 Foci scoring using fluorescence microscopy

3.6.2.1 Seeding of cells

Cells were seeded and treated on sterile coverslips placed in small petri dishes. Adherent cell lines were seeded at a density of 5000 cells per well in 2 ml of media and left for 24 hours to attach prior to treatment. PBLS were seeded immediately after isolation from the blood sample (section 3.3.2) at a density of $1 \times 10^6$ cells per well in 2 ml of QBL media and left for 72 hours to culture prior to performing an experiment. During this time a proportion of these suspension cells adhered to the coverslip.
3.6.2.2 Treatment with SN-38
The old media was removed, and for this assay, the PBLs were treated as adherent cells with 2 ml of final drug concentration (section 3.2.5).

3.6.2.3 Fixing of cells
The drug containing media was removed at the specified time point and the coverslips were gently washed twice with 2ml of ice cold PBS before cells were fixed in 2 ml of ice cold methanol and stored at -20°C overnight (or for a maximum of 72 hours) prior to immunostaining.

3.6.2.4 Immunostaining
The methanol was removed and the cells were rehydrated with 2 changes of PBS for 20 minutes, following which, 200 μl of blocking buffer (section 3.1.3) was added for 5 minutes. They were incubated in primary anti-phosphohistone H2AX mouse monoclonal antibody diluted in blocking buffer (1:200) at room temperature for 2 hours in the dark on a shaker. The coverslips were washed with 1 ml KCM washing buffer (section 3.1.3) 4 times and then incubated at room temperature in the dark on a shaker for 1 hour in 200 μl of the secondary Alexa Fluor™ goat anti-mouse IgG diluted 1:200 in blocking buffer. Samples were then washed with 1 ml KCM washing buffer for 5 minutes 4 times.
15 μl of SlowFade Gold™ antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI), pre-warmed to room temperature, was placed on the centre of labelled microscope slides. The coverslips were then removed from the petri dishes and mounted (cell coated side facing downwards) on the slides. The coverslips were secured at the corners with nail varnish and air dried at room temperature. Slides could then be stored at 4°C in the dark for up to 4 weeks until the cells were imaged.
3.6.2.5 Imaging and analysis

Fluorescence images were obtained using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Ltd) fitted with a 100 W mercury bulb at 400x magnification. Images were captured using an AxioCam HRc camera (Carl Zeiss Ltd). Analysis was performed using ImageJ version 1.42 software (research services branch of NIH).

Ten images (or a minimum of 40 cells in total) were analysed for each sample. Initially nuclei were identified and counted on the basis of DAPI staining with abnormally large (likely phase G2 or tetraploid) nuclei being excluded from the analysis (Figure 3-3). The number of foci staining with the Alexa Fluor™ was calculated using the particle counter function on imageJ. Results are presented as the average number of foci per cell.

Figure 3-3. γ-H2AX image analysis using fluorescence microscopy. Alexa Fluor™ Image has been superimposed on DAPI image using imageJ software.
3.6.3 Intensity based analysis using flow cytometry

3.6.3.1 Treatment and fixing of cells
Cells were treated and fixed in 70% ethanol as described in section 3.4 when performing cell cycle analysis. Following addition of the ethanol they were either kept on ice for 30 minutes and processed immediately or kept at -20°C for a maximum of a week.

3.6.3.2 Immunostaining
Samples were centrifuged at 1500 rpm for 5 minutes at 4°C, then washed in 1 ml of cold PBS and centrifuged again. Cells were then resuspended in 1 ml of cold PBST and placed on ice for 10 minutes. The PBST was removed by a further centrifugation and then samples were incubated in 200 μl of primary anti-phosphohistone H2AX mouse monoclonal antibody diluted in PBST (1:500) at room temperature, for 2 hours, in the dark, on a shaker. 1 ml of PBST was added, samples spun and the supernatant discarded. The cells were then incubated in 200 μl of Alexa Fluor™ goat anti-mouse IgG diluted 1:200 in PBST, at room temperature, for 1 hour, in the dark, on a shaker. Cells were washed with 1 ml PBS, centrifuged and resuspended in 500 μl of 50 μg/ml PI in PBS then analysed immediately on the FACS machine (Becton Dickinson, San Jose, CA) (section 3.4.1).

3.6.3.3 Flow cytometry analysis
Flow cytometry analysis was performed as illustrated in Figure 3-4.
Figure 3-4. Example of the processes involved in cell selection for γ-H2AX selection using flow cytometry.
A) The cell cycle distribution was determined as detailed in Figure 3-1. A third gate (R3) was then applied to select only those cells in G_0 and G_1.
B) The fluorescence intensity (FLH-1) in arbitrary units of 1 x 10^4 cells was plotted in histograms. The relative mean fluorescence per cell was calculated using CellQuest software.

3.7 Apoptosis assays

Two different apoptosis assays were used in this study namely: flow cytometric measurement of cellular DNA content and the annexin assay.

3.7.1 Measurement of cellular DNA content

Apoptotic cells have fragmented DNA that can be detected as a sub-G_1 peak when they are processed according to the flow cytometry protocol for cell cycle analysis (section 3.4). Cell cycle analysis was therefore performed and the size of the sub-G_1 peak, as detected by ModFit software, was indicative of the proportion of cells undergoing apoptosis (Figure 3-5).
3.7.2 Annexin Assay

3.7.2.1 Principles of the annexin assay

The annexin assay detects apoptosis by virtue of the fact that annexin V binds preferentially to phosphatidylserine (a phospholipid located on the inner cell membrane in viable cells). Annexin V is unable to bind to live cells since it is not capable of penetrating the cell membrane, however, in early apoptosis phosphatidylserine is externalised thus annexin may bind. Based on this phenomenon and by conjugating fluorescein-5-isothiocyanate (FITC) to annexin V, it is possible to analyse apoptotic cells by flow cytometry (section 3.4.1). In order to distinguish early from late apoptosis PI is used. An intact cell membrane is impermeable to PI and thus...
PI can only stain the DNA within cells during late apoptosis or necrosis when the cell membrane integrity is lost (van Engeland et al., 1998).

### 3.7.2.2 Annexin assay method

The Human Annexin V-FITC Apoptosis Kit (Bender MedSystems) was used. After treatment (sections 3.2.5 and 3.3.4) cells were transferred to pre-labelled FACS tubes, centrifuged for 5 minutes at 1500 rpm, 4°C and the drug containing supernatant was discarded. Samples were washed in 1 ml cold PBS, then following repeat centrifugation, they were resuspended in 1 ml 1 X annexin buffer (supplied as 5 X concentration diluted in water), to which was added 5µl of annexin V conjugate and the samples vortexed, then incubated at room temperature in the dark for 10 minutes. Samples were stained by the addition of 10µl PI (20 µg/ml) placed on ice and analysed immediately by flow cytometry as illustrated in Figure 3-6.
Figure 3-6. Example of the processes involved in cell selection during analysis of the annexin assay using flow cytometry

A) Cells were gated and cell debris was excluded according to the SSC and FSC parameters. $1 \times 10^4$ cells were analysed for each sample.
B) A quadrant box plot was produced with green fluorescence (annexin V staining) on the Y axis and red fluorescence (PI staining) on the X axis. The percentage of cells in each quadrant was calculated using CellQuest software. The cells in each quadrant were as follows:
- Left lower quadrant (LLQ). Annexin V and PI negative = viable cells.
- Left upper quadrant (LUQ). Annexin V positive, PI negative = early apoptosis.
- Right upper quadrant (RUQ). Annexin V and PI positive = late apoptotic or necrotic cells.
- Right lower Quadrant (RLQ). Annexin V negative, PI positive = debris.

3.8 Pharmacogenetic studies

3.8.1 Preparation of the DNA

DNA was extracted from PBLs using the Blood & Cell Culture DNA midi Kit from QIAGEN (Crawley, UK). The enzymes and buffers were part of the supplied kit and these were all equilibrated to room temperature before use, apart from buffer C1 which was kept on ice. All centrifuging during this procedure was performed at 4°C.
Aliquots of PBLs (total counts 5 – 10 x 10^6) were taken from the -80°C freezer and rapidly thawed in a water bath pre-heated to 37°C. Cells were pelleted by centrifugation at 1800 rpm for 5 minutes, then washed in 1 ml PBS and transferred to 15 ml falcon tubes. They were centrifuged again at 1500 rpm for 5 minutes then resuspended in 2 ml PBS to which was added 2 ml buffer C1 and 6 ml ice cold ultra pure distilled water. Samples were incubated on ice for 10 minutes and then these lysed cells were centrifuged at 3000 rpm for 15 minutes. These pelleted nuclei were resuspended by vortexing in 1 ml buffer C1 and 3 ml ultrapure water and were then centrifuged again at 3000 rpm for 15 minutes. 5ml of buffer G2 was added and the nuclei were resuspended as thoroughly as possible by vortexing for 30 seconds at maximum speed. 95 µl of protease was added and samples left to incubate at 50°C for 60 minutes.

After incubation the samples were vortexed for 10 seconds and applied to an equilibrated 100/G Qiagen Genomic-tip (equilibration was carried out using 4 ml buffer QBT) and allowed to move through by gravity flow. The Qiagen genomic-tip was subsequently washed with 2 x 7.5 ml of a wash buffer QC. The genomic DNA was finally eluted with 5 ml of elution buffer QF. To precipitate the DNA, 3.5 ml of isopropanol was added and the samples were centrifuged at 3000 rpm for 15 minutes. This DNA pellet was washed with 2 ml of ice-cold 70% ethanol, vortexed briefly, re-centrifuged and air-dried. It was then dissolved in 100 µl of 1 x TE on a shaker overnight at 4°C. Samples were then stored at -20°C until analysis.

3.8.2 DNA quantification

The DNA concentration was determined using a ND-1000 low volume cuvette free spectrophotometer (NanoDrop Technologies). This machine was initially blanked with
1.5 µl of water and then calibrated using 1.5 µl of 1 x TE. Quantification was performed by loading 1.5 µl of DNA and then measuring the UV absorbance at 260 nm and 280 nm. The calibration of the machine was such that an absorbance of 1 at A$_{260}$ corresponded to 50 µg/ml DNA. The purity was assessed by the ratio A$_{260}$/A$_{280}$ aiming for a ratio of 1.8 being taken as pure DNA.

3.8.3 DNA sequencing

The UGT1A1 variable length (TA)$_n$ repeat polymorphism (UGT1A1*28) was assessed using sequencing technology.

3.8.3.1 Principles of automated fluorescence sequencing by capillary electrophoresis

Sequencing reactions are analogous to PCR reactions but only use one primer. The reaction mix differs in that it contains 95% regular nucleotides and 5% dideoxynucleotide triphosphates (ddNTP); ddNTPs do not possess a 3' hydroxyl group, therefore once one is added to the end of a DNA strand, there's no way to continue elongation and the strand will terminate. Most of the time a normal nucleotide is incorporated but 5% of time a ddNTP will be added and the chain will terminate. These DNA fragments formed are of variable size and so can be separated by electrophoresis. If each ddNTP is given a fluorescent label the DNA sequence can then be determined using an automated fluorescent sequencer (Applied Biosystems, 2009) (Figure 3-7).
3.8.3.2 DNA sequencing method

Briefly, this process involved: a) polymerase chain reaction (PCR) of the sequence of interest, b) quantification of PCR product by agarose gel analysis, c) clean up of this product, d) carrying out of the sequencing reaction and e) clean up of sequencing product. This final product was processed by automated fluorescent sequencing by capillary electrophoresis on a 3730 DNA analyser (Applied Biosystems) at the PNACL facilities at the University of Leicester.

1) PCR

PCR was performed in a designated area to avoid contamination. Primers, namely 5′-TATCTCTGAAAGTGAACTC-3′ (sense) and 5′-ATCAACAGTATCTTCC CAG-3′ (antisense), were purchased from biomers.net. These were the same as those used by Carlini et al. (Carlini et al., 2005). They were also confirmed on the University of California Santa Cruz genome browser (http://genome.ucsc.edu/) to amplify a 254 base pair region of the UGT1A1 gene.

Primers were diluted with 1 x Tris/EDTA to a stock concentration of 100 pmol/µl and then further diluted with filtered double distilled water to a 10 pmol/µl working
solution. Primers were stored at -20°C and the working solution was thawed a maximum of 10 times prior to fresh being made to prevent degradation due to repeated freeze thawing.

The reaction for each PCR sample was prepared in either 96-well plates or PCR tubes on ice (ABgene). This reaction was: ~ 20 ng DNA (in a 1 µl volume thus the genomic DNA was further diluted in ddH2O as necessary), 0.3 µl sense primer, 0.3 µl antisense primer, 0.5 µl Taq polymerase and 0.9 µl 11.1 x PCR buffer. This was then made up to a volume of 10 µl using dd H2O. A negative control (consisting ddH2O instead of DNA being added to the PCR reaction mix) was analysed with each PCR cycle performed. PCR was carried out in a PTC-220 Thermo Cycler (MJ research). The optimised PCR profile was as follows:

- 1 x 10 minute denaturation at 91°C
- 35 cycles of: denaturation for 1 minute at 91°C, annealing for 1 minute at 45°C and extension for 1 minute at 72 °C
- 1 x 5 minute final extension at 72 °C
- 4°C until removal from the block.

The PCR product was stored at -20°C until analysis

2) Quantification of PCR product

A 2% agarose gel was made by dissolving 2 g type 1 agarose in 100 ml 1 x TBE (section 3.1.3) by microwaving until all had dissolved. This was then allowed to cool and once the gel was 60 °C it was poured, taking care to avoid air bubbles, and allowed to set for at least 1 hour until electrophoresis was performed.
3 µl of PCR product was added to 3 µl of 6 x loading buffer and 12 µl of water and then added to the wells in the gel. The gel was electrophoresed for 2 hours at 100 V using 1 x TBE as the electrophoresis buffer. 6 µl of a 50 bp DNA ladder (New England BioLabs) was ran at the edge of the gel.

The DNA was stained by immersing the gel in 100 µl of 1 x TBE with 20 µl of Nancy-520 DNA gel stain for 1 hour in the dark on a shaker.

The DNA bands were visualised using GeneSnap software on the Chemogenius Bioimaging System (Syngene). In the event of a band being visualised in the negative control the PCR products were discarded and the procedure repeated with fresh reagents as this indicated likely DNA contamination.

The size and quantity of DNA fragments were estimated by comparing the position and brightness of the bands to that of the known size and concentration in the DNA ladder.

3) Clean up of PCR product

5 µl of the PCR product was added to 0.5 µl of exonuclease I (exo 1) and 1.5 µl of Shrimp Alkaline Phosphatase (SAP). This was then placed in the PCR machine at 37°C for one hour followed by a 15 minute incubation at 80°C to inactivate the enzyme. This was then held at 15°C until the sample was removed from the block

4) Sequencing reaction

A 10 µl final volume was prepared by adding:

- 1 µl big dye terminator ready reaction mix
- 1.5 µl big dye terminator buffer
- ~100 ng PCR product template (minimum 20 ng, maximum volume 6.5 µl)
- 1 µl sense primer
ddH2O to make up final volume of 10 µl

This was then sequenced in the PCR machine with 25 cycles of the following 3 steps:

- 96 ºC for 10 seconds
- 50 ºC for 5 seconds
- 60 ºC for 4 minutes

5) **Clean up after sequencing**

Mastermix was made containing 10 µl ddH2O and 2 µl 2.2% sodium dodecyl sulfate (SDS) per sample. An aliquot of 12 µl was added to each reaction, thoroughly mixed and then cycled in the PCR Machine at 98ºC for 5 minutes, followed by 25ºC for 10 minutes.

The dye was removed immediately following clean up using performa gel filtration spin columns (EdgeBio). These were spun at 3400 rpm for 3 minutes, the collection tube was then discarded and the gel column moved into a fresh tube. The DNA sample was added carefully to the gel column prior to centrifuging again at 3400 rpm for 3 minutes. Samples were labelled and then sent to PNACL for processing. Samples were stored at -20 ºC prior to processing.

6) **Viewing sequencing data**

The final electropherogram (sequence trace) was viewed using Sequence Scanner version 1.0 (Windows XP and Windows 2000). The number of TA repeats were manually counted (Figure 3-8).
3.8.4 Restriction fragment length polymorphism analysis.

This method was used to detect UGT1A1*93 (i.e. 3156G>A SNP in the UGT1A1 promoter).

3.8.4.1 PCR of the product of interest

Primers were designed using the UCSC genome browser. The PCR product was 359 base pairs long. These primers were: 5’ – TAACCTGAAACCGGACTT - 3’ (sense) and 5’ - CACCACCCTGTTGACGCTTCC - 3’ (antisense).

The PCR reaction was set up using the method described in section 3.8.3.2 with the only difference being that the annealing temperature for this primer set was 58°C. The PCR product was then viewed on an agarose gel as detailed in section 3.8.2.
3.8.4.2 Enzyme digest of the PCR product

The DdeI restriction endonuclease was identified using NEBcutter (NEB) to cut at the following sequence:

\[ 5' - C\text{TNA}_G - 3' \]

Thus, this enzyme would digest the PCR product DNA into 2 fragments (199 and 160 base pairs long) only if the G > A SNP was present.

The digest reaction was set up in PCR tubes as follows: 5 μl PCR product, 5 μl buffer 3 (NEB), 2 μl DdeI (NEB) and 38 μl of water. This was incubated at 37°C in the PCR machine and then stored at -20°C until imaged.

3.8.4.3 Imaging of digest product

30 μl of digest product was added to 6 μl of 6 x loading buffer then electrophoresed and imaged on a 2% agarose gel using the method described in 3.8.2.

3.9 High performance liquid chromatography-fluorescence detection and liquid chromatography-mass spectrometry

3.9.1 Cell treatments

PBLs (5 x 10^6 cells) cultured for 72 h in QBL media were treated with either 50 μM irinotecan or 0.05 and 5 μM SN-38 for 1, 4, 6 or 24 hours. DMSO was used as the control and the amount present in all the treatments was 0.20%.

3.9.2 Cell harvesting and methanol extraction

Following treatment, samples were centrifuged at 1500 rpm for 5 min at 4°C and then washed twice with 1 mL of ice-cold PBS. Pelleted cells were lysed by the addition of 1 mL of ice cold methanol and evaporated to dryness, overnight, at room temperature.
using a Savant SpeedVac SS210A centrifugal evaporator (Thermo Fisher Scientific Inc). Dried samples were stored at -80°C until being further processed as described below.

3.9.3 Chloroform-methanol extraction of cell pellets.

The methanol quenched dried cell pellet samples were placed on ice and to each sample was added 300 μL of CHCl₃/methanol (2:1, v/v) which was mixed thoroughly by vortexing for 30 seconds. The samples were then centrifuged at 16000 g in a Heraeus Fresco 21 centrifuge (Thermo Electron Corporation) for 10 minutes, resulting in the formation of three layers: an upper aqueous layer containing the methanol and drug metabolites, an interphase layer containing proteins and debris and a lower organic layer containing lipids. The aqueous fraction was transferred to a new eppendorf. The middle layer was mixed with a further 300 μL of CHCl₃/methanol (2:1, v/v) and the extraction process repeated. The organic layer was discarded. The double extraction was employed to maximise the recovery of the metabolites. The aqueous fractions were evaporated to dryness overnight in the SpeedVac at room temperature and stored at -80°C until being analysed.

3.9.4 Preparation of standard and cell extract solutions

Stocks solutions of both SN-38 and irinotecan dissolved in DMSO (section 3.1.4) were used to prepare standard solutions with concentrations of 0.1 pmol/μL and 1 pmol/μL by dilution with 20 mM ammonium acetate pH 3.5/acetonitrile (20:80, v/v). The extracted cell samples were dissolved in 200 μL of 20 mM ammonium acetate pH 3.5/acetonitrile (20:80, v/v) and 10 μL aliquots were injected onto the HPLC-fluorescence (HPLC-FL) system or liquid chromatography-mass spectrometry (LC-MS) system as described below.
3.9.5 High performance liquid chromatography-fluorescence detection

3.9.5.1 Principles of HPLC

HPLC is a widely used separation technique.Briefly, it involves the injection of a liquid sample into a column packed with small particles (stationary phase); the most commonly used columns contain a chemically modified silica stationary phase in which a C_{18} alkyl group is bonded to the silica surface (Ardrey, 2003). The individual components of the sample bind to the column but are moved down with a liquid (mobile phase) forced through at a constant rate by high pressure delivered by a pump. Differences in polarity between the components of the sample ensure that they elute from the column at different time points. These components can then be identified as they elute using a range of detectors (e.g. UV, fluorescence, refractive index or spectroscopy detectors) (Robards et al., 1994).

3.9.5.2 Equipment and chromatographic conditions

The HPLC method used in this study was adapted from the method described by Guo et al. with some modifications (Guo et al., 2003). The samples were analysed using a Varian ProStar analytical HPLC system (Agilent Technologies formerly Varian) which consisted of a ProStar 230 solvent delivery module, a ProStar 410 autosampler with 100 μL injection loop and an integral column oven maintained at 35 °C. A 10 μL aliquot of each sample or standard solution was injected on to a Hypersil C_{18} HyPurity, 15 cm x 2.1 mm, 3 μm analytical column (Thermo Scientific) plus guard column (1 cm x 2.1 mm, 3 μm) and Krudkatcher filter (0.5 μm, Phenomenex, Macclesfield, UK). Fluorescence was monitored using a 470 scanning fluorescence detector (Waters Ltd.) which had excitation and emission wavelengths set at 368 nm and 515 nm, respectively. The
mobile phase consisted of solvent A, 20 mM ammonium acetate pH 3.5 (adjusted to pH 3.5 with 5 M HCl) and solvent B, acetonitrile. Prior to usage the aqueous mobile phase solvent was filtered through a 0.45 μm cellulose-nitrate membrane filter (Fisher Scientific) under vacuum. The flow rate was 0.2 mL/min and the following gradient was used to elute the column; 0 min- 25% B, 5 min -25% B, 15 min – 45% B, 20 min – 65% B., 20.1 min – 25 % B and 35 min – 25% B. The data was processed using the Star chromatography work-station software (version 6.20, Varian).

3.9.6 Liquid chromatography-mass spectrometry (LC-MS)

This work was kindly performed by Dr Raj Singh in the Department of Cancer Studies and Molecular Medicine at the University of Leicester.

The LC-MS system consisted of a Waters Alliance 2695 separations module with a 100 μL injection loop connected to a Micromass Quattro Ultima Pt. (Waters Ltd, Manchester, UK) tandem quadrupole mass spectrometer with an electrospray ionisation interface. The temperature of the electrospray source was maintained at 110 °C and the desolvation temperature at 350 °C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas (25 L/h). The capillary voltage was set at 3.00 kV. The cone and RF1 lens voltages were 40 V and 60 V, respectively. The photomultiplier was set at 850 V. The mass spectrometer was tuned by using a standard solution of SN-38 which was diluted to a concentration of 100 pmol/μL using 0.1% formic acid/acetonitrile (75:25, v/v) from a stock solution dissolved in DMSO and introduced by continuous infusion at a flow rate of 10 μL/min with a Harvard model 22 syringe pump (Havard Apparatus Ltd., Edenbridge, UK).

A 10 μL aliquot of each sample or standard was injected on to a Hypersil C\textsubscript{18} HyPurity, 15 cm x 2.1 mm, 3 μm analytical column plus guard column (1 cm x 2.1 mm, 3 μm) and
Krudkatcher filter (0.5 µm). The column was located in the column oven and maintained at 35 °C. The mobile phase consisted of solvent A, 0.1% formic acid and solvent B, acetonitrile. The flow rate was 0.12 mL/min and the following gradient was used to elute the column; 0 min - 22% B, 5 min - 22% B, 15 min – 45% B, 20 min – 65% B., 20.1 min – 22 % B and 35 min – 22% B.

The samples were analysed in positive ionization tandem mass spectrometry (MS/MS) selected reaction monitoring (SRM) mode for the [M+H]+ ion to major fragment transitions of irinotecan m/z 587 to 543, SN-38 m/z 393 to 349 and SN-38 G m/z 569 to 393. The collision gas was argon (indicated cell pressure 2.0 × 10⁻³ mbar) and the collision energy set at 21 eV. The dwell time was set to 200 ms and the resolution was 1.5 m/z units at peak base. The data was processed using MassLynx software (version 4.1, Waters Ltd.).

3.10 Clinical study design

3.10.1 Patient recruitment

Ethical approval was sought and obtained from the Nottingham Research Ethics Committee (REC) 1 (REC reference number 09/H0403/8) in March 2009. University Hospitals of Leicester NHS Trust (UHL) Research and Development (R and D) approval was granted in April 2009. Trial participants were identified as those who were due to receive second-line irinotecan based chemotherapy for metastatic CRC at LRI. Both male and female patients were eligible for this study providing they were over 18 years of age and able to give written informed consent. All consenting patients who met these inclusion criteria being treated between September 2009 and May 2011 were included in this study.
3.10.2 Blood sampling and isolation of PBLs

Initially, 3 x 10ml blood samples in heparinised vials (SARSTEDT) were obtained per patient. The timing of these samples was a) before, b) 1 hour after and c) 24 hours after chemotherapy. Ideally bloods were obtained on the first cycle of treatment however, if this was not achieved, samples were obtained at a subsequent cycle. Such cases are identified in the results. Following an interim analysis in November 2010, a substantial amendment was made to the trial protocol so that only 1 x 20 ml blood sample was obtained before chemotherapy.

Samples were coded and kept at room temperature and PBLs were isolated as quickly as possible following venepuncture as described section 3.3.2.

Between 2.5 – 5 x 10⁶ PBLs (depending on the number isolated) from the pre sample were cultured in QBL media (section 3.3.3). A single 25 cm² flask was cultured for patients prior to the interim analysis and 2 flasks were cultured thereafter. All other PBLs were frozen in freezing media in a minimum of 5 x 1ml aliquots per sample (3.3.2)

3.10.3 In vivo ACA

This experiment was only performed on 21 of those participants recruited prior to the interim analysis. One aliquot of frozen PBLs from each time point was rapidly thawed, cells counted, slides prepared and the ACA performed as described in section 3.5. All three samples (pre, 1 hour and 24 hours post) from each patient were processed simultaneously in the same electrophoresis tank.

3.10.4 Ex vivo ACA

This assay was only performed on those PBLs that were isolated from the blood sample taken prior to chemotherapy and then cultured for 72 hours. Two experiments, a dose
response and a time course were performed for each participant. Cells were treated with SN-38 in 6-well plates as described in section 3.3.4. For the dose response experiment, cells were treated for 1 hour with 0 - 5 µM SN-38. For the time course experiment, cells were treated with 5 µM SN-38 for 1, 4 and 10 hours. The final DMSO concentration across all samples was 0.196%. When running the ACA only 1 patient was analysed per electrophoresis tank.

3.10.5 ACA Controls

HT-29 cell controls were processed alongside the clinical samples. For the in vivo experiments, pre prepared aliquots of untreated and 10 Gy irradiated HT-29 cells were used. These cells had been cultured and irradiated at the same time and then stored in 750 µl aliquots at -20°C until required for use (3.2.6). Single aliquots of these control cells were rapidly thawed and then 3 x 250µl aliquots were pipetted into 3 eppendorfs and centrifuged at 0.3 x g, 4 °C for 5 minutes g in a Heraeus Fresco 21 centrifuge (Thermo Electron Corporation). The freezing media was discarded prior to the addition of LMP agarose to make slides.

For the in-vitro experiments, there was one 0.196% DMSO only negative control and two positive controls which were treatment with either 1 µM SN-38 for 1 hour or 10 Gy irradiation. The SN-38 treatment of HT-29 cells was performed alongside the treatment of PBLs. The irradiated cells were those that had been pre-prepared and were thawed from the freezer as above.
3.10.6 Measurement of γ-H2AX

γ-H2AX was measured in 2 patients using the foci method (section 3.6.2) and 7 patients using the flow cytometry method (section 3.6.3). For each participant a dose response and time course was performed.

3.10.7 Pharmacogenetic studies

UGT1A1*28 and *93 polymorphisms were assessed in all participants (method described in section 3.8)

3.10.8 Clinical data

Clinical data was obtained by reviewing the patients’ notes. Baseline information recorded included the following:-

- Age
- Sex
- Performance status
- Routine blood parameters (white cell count, bilirubin)
- Cycle number sample taken
- Dose of sample taken

Data about the administration of treatment recorded was as follows:

- Total number of cycles received
- Toxicities experienced. Toxicities were graded according to the Common Toxicity Criteria (CTC) Version 4 (2009).
- Dose reductions and delays
- Hospital admissions and reasons.
Data recorded about the response to treatment was:

- Best response using the Response Evaluation Criteria In Solid Tumors (RECIST) criteria (Eisenhauer et al., 2009).
- Progression free survival
- Overall survival

3.10.9 Statistical analysis

Statistical analysis was then performed using PASW statistics 18.0 for Windows. The normality of distributions was assessed from probability plots.

For the ACA results, the toxicity, response, survival and polymorphism data were correlated with the percentage tail DNA. Where statistical values are shown, unless otherwise stated the independent samples t-test was used for normal data and the Mann-Whitney U test was performed for non parametric data. Correlations were assessed using the Pearson correlation co-efficient. The survival endpoints were correlated with the ACA data by using the log rank test. P values are significant at <0.05
4 Clinical trial participant data: baseline characteristics, toxicities and response to irinotecan treatment.

An analysis investigating patient demographics, disease characteristics, genotypes and treatment effect was performed prior to embarking on the interpretation and correlation of laboratory DNA damage measures with the clinical findings. The aims of this analysis were to determine:

A) Whether the study participants were representative of the metastatic colorectal cancer population.

B) If any baseline parameters correlated with irinotecan treatment outcome.

C) If this group of patients would have benefited from a predictive test of irinotecan effect.

D) Whether any major differences between patients grouped according to the presence of toxicities or response to irinotecan treatment (that could potentially confound future data analysis) existed.

E) Whether UGT1A1*28 predicted outcome in this patient population.

4.1 Patient recruitment and data collection

Forty-two patients, due to receive 2nd line irinotecan based chemotherapy, were recruited between April 2009 and May 2011. Clinical data collection was concluded in August 2011 at which point 39 (93%) patients had completed or stopped their irinotecan treatment and 29 (69%) had died. Of the 14 patients that were still alive when data collection was terminated, 9 had confirmed disease progression with only 5 still having stable or responding disease. This follow up time was thus sufficient to
obtain toxicity data for all participants and to allow median survival times to be calculated.

It is noteworthy that following detection of disease progression, over a fifth of the participants received further cancer treatment, which may potentially have confounded the overall survival data analysis.

4.2 Samples acquired

Blood samples were obtained prior to the first cycle of chemotherapy in 22 patients, the remainder were obtained on subsequent cycles: 9 on cycle 2, 5 on cycle 3, 2 on cycle 4 and 1 each on cycles 6, 7, 10 and 11 (Table 5-1 and Table 7-1).

4.3 Chemotherapy regimen

Forty-one patients (98%) received irinotecan in combination with a fluoropyrimidine: 39 as part of the biweekly FOLFIRI regimen at a starting dose of 180 mg/m$^2$, 1 received FOLFIRI with a reduced starting dose of 135 mg/m$^2$ and 1 received 3 weekly capecitabine/irinotecan treatment at a dose of 250mg/m$^2$. Two of these patients received bevacizumab in addition to the FOLFIRI and 12 received FOLFIRI combined with either an oral endothelin receptor antagonist (ZD4054) or a placebo as part of the FOLFERA study. Only a single patient who was intolerant to 5-FU was treated with irinotecan monotherapy at the higher dose of 350mg/m$^2$ every 3 weeks (Figure 4-1).
Figure 4-1. A pie chart illustrating the proportion of trial participants who received each irinotecan based regimen. In the FOLFERA study, patients received FOLFIRI in combination with either an oral endothelin receptor antagonist (ZD4054) or a placebo.

It is noteworthy that the variety of treatment regimens used may have confounded both the final laboratory and clinical data analysis. Fluopyrimidines may have increased the DNA damage levels, by altering the stability and structure of DNA and by interfering with its repair (section 1.5.3.1), although such an effect has not been previously described in the literature (summarised in section 1.8.3.1.1). Any potential effect of ZD4054 on DNA damage and repair was also unknown. In addition when analysing the clinical data, as fluoropyrimidines and irinotecan are both associated with neutropaenia and diarrhoea, it was not possible to confirm which of these drugs was responsible for the toxicities documented in the trial participants.
4.4 Patient characteristics and their association with toxicities and response to irinotecan treatment.

The general demographics of all trial participants and of individuals grouped according to the subsequent development of grade 3/4 toxicities (diarrhoea and neutropenia) and response to treatment are summarised in Table 4-1. These demographics were similar to those reported in large, multicentre clinical studies (Seymour et al., 2007), (Maughan et al., 2011) and thus this group was considered to be representative of the metastatic CRC population. In general, patient characteristics were well balanced within both the toxicity and response sub-groups with there being no significant difference in either age (p=0.362 and 0.276 respectively calculated using the Mann Whitney U test) or sex (p = 0.958 and 0.679 calculated using the Chi-Squared test). The notable exception was that those with toxicities were significantly more likely to have a poorer performance status (PS) than those who tolerated treatment well (p=0.017 calculated using the Chi-squared test for trend). This was unsurprising as the association of poor PS with toxicities is well recognised (Kweekel et al., 2008a). There was also a trend that those with poorer PS were more likely to experience progressive disease however this did not reach significance (p=0.145).
Table 4-1. Baseline demographics of all clinical trial participants and the corresponding data when patients were grouped according to the development of grade 3/4 toxicities (diarrhoea and neutropenia) and response to treatment

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Toxicity groups</th>
<th>Response groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤ Grade 2</td>
<td>Grade 3 - 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>toxicities</td>
<td>toxicities</td>
</tr>
<tr>
<td>Number of assessable patients</td>
<td>42 (100%)</td>
<td>31 (74%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (64%)</td>
<td>20 (65%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>Female</td>
<td>15 (36%)</td>
<td>11 (35%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>Median age (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64 (34 – 77)</td>
<td>62 (34 – 77)</td>
<td>67 (61-74)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>39 (93%)</td>
<td>28 (91%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (5%)</td>
<td>2 (6%)</td>
<td>0</td>
</tr>
<tr>
<td>Afro-Caribbean</td>
<td>1 (2%)</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>ECOG PS at baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>17 (40%)</td>
<td>16 (52%)</td>
<td>1 (9%) *</td>
</tr>
<tr>
<td>1</td>
<td>23 (55%)</td>
<td>14 (45%)</td>
<td>9 (82%)</td>
</tr>
<tr>
<td>2</td>
<td>2 (5%)</td>
<td>1 (3%)</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

* = statistically significant with p<0.05 calculated using the Chi-squared test for trend
# = 6 patients did not have response assessed due to the premature cessation of treatment as a result of toxicities or death.

Table 4-2 illustrates the baseline blood parameters and disease characteristics of all clinical trial participants and once again the corresponding data when patients were grouped according to the development of grade 3/4 toxicities (diarrhoea and neutropenia) and response to treatment are shown. A trend was observed that those with hepatic metastasis were less prone to toxicities than those with extra-hepatic metastasis only, although this did not reach significance (p=0.084 calculated using the Chi-squared test). Data demonstrating the association of routine blood parameters with irinotecan effect, reported in the past, has been conflicting (section 1.7.2.1). In this study neither baseline bilirubin nor white cell count (WCC) demonstrated any correlation with response or toxicities to irinotecan treatment.
Table 4-2. Baseline blood parameters and disease characteristics of all clinical trial participants and the corresponding data when patients were grouped according to the development of grade 3/4 toxicities (diarrhoea and neutropenia) and response to treatment.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Toxicity groups</th>
<th>Response groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤ Grade 2</td>
<td>Grade 3 - 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>toxicities</td>
<td>toxicities</td>
</tr>
<tr>
<td>Baseline WCC (x10^9/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 x 10^9 per L</td>
<td>37 (88%)</td>
<td>26 (84%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>≥10 x 10^9 per L</td>
<td>5 (12%)</td>
<td>5 (16%)</td>
<td>0</td>
</tr>
<tr>
<td>Baseline Bilirubin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 μmol/L</td>
<td>27 (64%)</td>
<td>20 (65%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>10 – 20 μmol/L</td>
<td>13 (31%)</td>
<td>9 (29%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>&gt;20 μmol/L</td>
<td>2 (5%)</td>
<td>2 (6%)</td>
<td>0</td>
</tr>
<tr>
<td>Status of primary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resected</td>
<td>17 (40%)</td>
<td>10 (32%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>Unresected</td>
<td>22 (52%)</td>
<td>19 (61%)</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Local recurrence</td>
<td>3 (7%)</td>
<td>2 (6%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Site of metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locally advanced</td>
<td>3 (7%)</td>
<td>3 (10%)</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>5 (12%)</td>
<td>4 (13%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Liver + others</td>
<td>23 (55%)</td>
<td>19 (61%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>None liver</td>
<td>11 (26%)</td>
<td>5 (16%)</td>
<td>6 (5%)</td>
</tr>
<tr>
<td>Metastatectomy peri-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>irinotecan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (7%)</td>
<td>2 (6%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>No</td>
<td>39 (93%)</td>
<td>29 (94%)</td>
<td>10 (91%)</td>
</tr>
</tbody>
</table>

NB/ There were no significant differences between the toxicity and response subgroups calculated using the Chi-squared test.

Overall these data confirmed, as anticipated from the published literature, that it was not possible to accurately predict irinotecan effect for each individual using these baseline characteristics alone. They also demonstrated that the sub-groups used when assessing toxicities and response were of equivalent age, sex and disease status; thus with the exception of PS there would be no confounding variables when using these sub-groups to analyse laboratory indices of DNA damage.
4.5 Investigating the requirement for a predictive test of toxicities and response to irinotecan treatment in the study population.

In keeping with the toxicity rates from larger clinical studies (Table 1-2), 11 patients (26%) experienced grade 3/4 toxicities and thus would have benefited from commencing treatment at a lower dose or using an alternative drug:

- 2 had grade 3/4 neutopenia only
- 5 had grade 3/4 diarrhoea only
- 4 had both grade 3/4 neutropenia and diarrhoea

Of the 6 patients with neutropenia, 4 developed neutropenic sepsis and 3 of these cases were also associated with severe diarrhoea. Four patients with severe toxicities received only 1 cycle of irinotecan chemotherapy: 2 of these died of neutropenic sepsis following their first cycle and an additional 2 experienced a decline in performance status following the toxicities experienced and were therefore deemed medically unfit to receive further treatment. An additional 3 patients experienced grade 3/4 fatigue which in 1 individual was also associated with severe nausea and vomiting.

Of those who were assessable for response, 7 (19%) derived no clinical benefit with the best response being progressive disease (PD). Only 1 patient with PD also experienced grade 3/4 neutropenia or diarrhoea however, 3 patients with PD did report grade 3 fatigue. This illustrates the difficulty in using fatigue as a sign of toxicity as this symptom may be a manifestation of both the cancer and/or the treatment. For this reason, when conducting statistical analysis, only life threatening toxicities (severe neutropenia and diarrhoea) were used.
4.6 Survival data

Overall survival (OS) data were available for all 42 participants whereas progression free survival (PFS) was only assessed in 35 of the patients. The data are summarised in Figure 4-2. For all patients, the median PFS and OS were 200 days (~6.6 months) and 300 days (~10 months) respectively. This PFS was better than anticipated from previous studies in which second line FOLFIRI following oxaliplatin containing chemotherapy, was associated with a median PFS ranging from 2.5 to 6.2 months (Tournigand et al., 2004, Clarke et al., 2011). The OS however was comparable to other studies reporting median survival times ranging between 9.5 and 15.4 months (Graeven et al., 2007, Clarke et al., 2011). There was no significant difference in PFS between those with toxicities and those who tolerated treatment well however the OS was significantly less in those with toxicities compared to those without (median 100 versus 350 days). This confirmed the detriment that toxicities to chemotherapy are known to have on treatment outcome (section 1.7.1). Clearly, those who progressed on treatment had inferior survival; the median PFS and OS were 75 and 200 days respectively in those with PD compared to 275 and 325 days in those who had stable disease or partial response.
Figure 4-2. Kaplan-Meier plots for A) progression free survival and B) overall survival for all patients and patients classified according to toxicity and response. P values were calculated using the log rank test.
4.7 Investigating the association of UGT1A1*28 and UGT1A1*93 with irinotecan treatment outcome.

UGT1A1*28 is the most comprehensively investigated predictive test of irinotecan effect although its associations with response and toxicities to date have been conflicting (section 1.7.2.3.3). All patients were sequenced to determine whether this polymorphism was present. Representative electropherograms obtained are illustrated in Figure 4-3. In total, 21 patients were wild type homozygotes (UGT1A1*1*1), 6 were mutant homozygotes (UGT1A1*28*28) and 15 were heterozygotes (UGT1A1*1*28). These gene frequencies were in Hardy-Weinburg equilibrium (p=0.50 calculated using the Chi-Squared test).

In addition, the presence of UGT1A1*93 (another SNP reported to be associated with toxicities) was assessed using restriction fragment length polymorphism analysis. Six patients were homozygous for UGT1A1*93 with the entire PCR product digesting into 2 fragments 199 and 160 base pairs long (Figure 4-4). All of the 6 patients homozygous for UGT1A1*93 were also homozygous for UGT1A1*28. These two polymorphisms are known to exist in linkage disequilibrium (Innocenti et al., 2002) therefore the occurrence of both of them together confirmed that the detection of mutant homozygotes was robust.
Figure 4-3. Representative electropherograms obtained using automated fluorescence sequencing by capillary electrophoresis of A) UGT1A1 *1 (wild type) homozygotes, B) UGT1A1*28 homozygotes and C) UGT1A1 *1 *28 heterozygotes.
Figure 4-3 continued.

B) UGT1A1 *28 *28

Relative fluorescence

Fragment size (base pairs)
Figure 4-3 continued.
There were no significant associations of UGT1A1*28*28 with either toxicities or response to treatment although it was observed that all assessable patients with this genotype had at least stabilisation of disease but patient numbers were only small (n=5) (Table 4-3). The lack of predictive value of this polymorphism was in keeping with the largest meta-analysis of its value to date, which confirmed that its detection was most helpful for high doses of irinotecan as prescribed in the irinotecan monotherapy regimen. This regimen was administered to only a single patient in this study whilst the majority received combination treatment containing a lower dose of irinotecan, when UGT1A1*28 genotyping has been shown to be less useful (Hoskins et al., 2007) (section 1.7.2.3.3).
Table 4-3. Investigating the association of UGT1A1 *28 with toxicities and response to irinotecan treatment.

<table>
<thead>
<tr>
<th></th>
<th>Toxicity groups</th>
<th>Response groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ Grade 2</td>
<td>Grade 3 - 4</td>
</tr>
<tr>
<td>toxicities</td>
<td>toxicities</td>
<td></td>
</tr>
<tr>
<td>UGT1A1<em>1</em>1</td>
<td>14 (45%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>UGT1A1<em>1</em>28</td>
<td>12 (39%)</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>UGT1A1<em>28</em>28</td>
<td>5 (16%)</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

P values for were calculated using the Chi-Squared test and were as follows: Toxicity p= 0.567, response p=0.375.

The effect of UGT1A1*28 on survival was also investigated (Figure 4-5). Those patients homozygous for this polymorphism did seem more likely to have an improved overall survival (median survival not yet reached in the UGT1A1*28 homozygotes) but this was just below the level of significance (p=0.057). The apparent tendency for these patients to be more likely respond to treatment and have improved overall survival could theoretically be explained by the fact that they glucuronidate SN-38 more slowly than wild type individuals and therefore have the active metabolite circulating for longer (Gupta et al., 1994). However, this possible correlation has not been consistent across all studies (section 1.7.2.3.3), so may just be a chance finding due to the relatively small sample size.
Figure 4-5. Kaplan-Meier plots for A) progression free survival and B) overall survival for patients classified according to the presence of UGT1A1*28*28. P values were calculated using the log rank test.

The possible association of UGT1A1*28*28 with response, suggested that its presence may differentiate efficient and slow metabolisers of SN-38 in this sample population. These data therefore supported the concept that grouping patients by UGT1A1 status, in addition to response and toxicities, may provide additional information as to whether laboratory measures of DNA damage reflect SN-38 metabolism.

4.8 Conclusion

The demographics of the trial participants were in keeping with those in large multicentre studies of this tumour type, therefore any correlations with the laboratory data in the translational part of this research could be assumed to be potentially relevant to the whole metastatic CRC population. It was not possible to predict irinotecan effect from baseline characteristics alone. The most widely described predictive test developed to date, namely presence of UGT1A1*28*28 was not associated with toxicities and inconclusively correlated with response and survival. A
predictive test of irinotecan effect could have improved the management of 17 (40%) of the patients recruited. Over a quarter would have benefitted from a predictive test of toxicities – in particular 4 patients (almost 10%) had treatment terminated after only 1 cycle because of either death or a drop in performance status as a result of these toxicities. In addition, almost one fifth received treatment with no clinical gain and thus would have benefitted from a predictive test of irinotecan response.

The median overall survival in those with toxicities or progressive disease in this cohort, was short (100 – 200 days) thus emphasising the paramount importance of maintaining quality of life when treating patients nearing the end stages of their disease. More research is therefore warranted to improve the outcome for these patients.
The in vivo study results: Investigating DNA damage induced in PBLs following irinotecan treatment

5.1 Introduction

Preliminary data, previously generated in this laboratory, purported that small increases in DNA damage in PBLs (measured using the ACA) induced by irinotecan exposure in vivo correlated with toxicities to treatment (Smith et al., 2007). However, patient numbers were small (n=4), therefore the first stage of this study was to investigate whether these findings could be substantiated. If so, this would then provide proof of principle for using DNA damage as a biomarker of irinotecan effect.

5.2 Results and discussion

5.2.1 DNA damage induced by irinotecan exposure in vivo, measured using the ACA, in clinical trial participants.

This assay was performed on samples obtained from the first 21 of the 42 patients recruited to the clinical study. The results are summarised in Table 5-1. In general, DNA damage levels across all clinical samples were minimal compared to those of the irradiated controls that were processed alongside them (mean percentage tail DNA 4.36% versus 17.5%). This demonstrates that the low levels of DNA damage in the PBLs were real and not due to the assay underestimating the damage.

Collectively, there was no significant difference in the mean percentage tail DNA following either 1 hour or 24 hours of irinotecan exposure compared to baseline (Figure 5-1).
### Table 5-1. *In vivo* study results: the raw data and the calculated change in percentage tail DNA detected before and after irinotecan treatment as measured using the ACA

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Chemotherapy cycle number</th>
<th>Median % tail DNA at baseline (s.d.)</th>
<th>Median % tail DNA 1 hour post irinotecan (s.d.)</th>
<th>Median % tail DNA 24 hours post irinotecan (s.d)</th>
<th>Difference in % tail DNA at 1 hour from baseline</th>
<th>Difference in % tail DNA at 24 hours from baseline</th>
<th>Difference in % tail DNA of irradiated from unirradiated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>7</td>
<td>4.76 (2.58)</td>
<td>4.85 (0.68)</td>
<td>N/A</td>
<td>0.09</td>
<td>N/A</td>
<td>19.22</td>
</tr>
<tr>
<td>002</td>
<td>4</td>
<td>4.70 (0.88)</td>
<td>5.24 (2.15)</td>
<td>5.06 (1.86)</td>
<td>0.54</td>
<td>0.36</td>
<td>19.22</td>
</tr>
<tr>
<td>003</td>
<td>11</td>
<td>4.44 (2.06)</td>
<td>3.85 (0.85)</td>
<td>4.29 (0.79)</td>
<td>-0.59</td>
<td>-0.15</td>
<td>19.22</td>
</tr>
<tr>
<td>004</td>
<td>2</td>
<td>3.72 (0.32)</td>
<td>4.27 (0.99)</td>
<td>4.61 (0.80)</td>
<td>0.55</td>
<td>0.88</td>
<td>11.05</td>
</tr>
<tr>
<td>005</td>
<td>2</td>
<td>3.23 (0.99)</td>
<td>4.36 (0.14)</td>
<td>4.82 (0.63)</td>
<td>1.13</td>
<td>1.59</td>
<td>11.05</td>
</tr>
<tr>
<td>006</td>
<td>2</td>
<td>3.12 (0.48)</td>
<td>4.73 (1.09)</td>
<td>4.77 (0.66)</td>
<td>1.61</td>
<td>1.65</td>
<td>11.05</td>
</tr>
<tr>
<td>007</td>
<td>10</td>
<td>3.09 (0.96)</td>
<td>3.01 (0.81)</td>
<td>4.11 (3.08)</td>
<td>-0.07</td>
<td>1.02</td>
<td>15.57</td>
</tr>
<tr>
<td>008</td>
<td>6</td>
<td>3.78 (0.57)</td>
<td>3.02 (0.44)</td>
<td>3.09 (0.89)</td>
<td>-0.76</td>
<td>-0.69</td>
<td>15.57</td>
</tr>
<tr>
<td>009</td>
<td>1</td>
<td>3.35 (1.29)</td>
<td>3.76 (0.95)</td>
<td>3.34 (1.17)</td>
<td>0.41</td>
<td>-0.02</td>
<td>15.57</td>
</tr>
<tr>
<td>010</td>
<td>3</td>
<td>4.39 (0.74)</td>
<td>2.32 (0.59)</td>
<td>3.53 (0.83)</td>
<td>-2.07</td>
<td>-0.86</td>
<td>22.43</td>
</tr>
<tr>
<td>011</td>
<td>1</td>
<td>3.86 (0.72)</td>
<td>4.74 (0.04)</td>
<td>6.29 (1.48)</td>
<td>0.87</td>
<td>2.41</td>
<td>22.43</td>
</tr>
<tr>
<td>012</td>
<td>4</td>
<td>3.29 (0.11)</td>
<td>2.92 (0.74)</td>
<td>5.77 (0.34)</td>
<td>-0.37</td>
<td>2.5</td>
<td>22.43</td>
</tr>
<tr>
<td>013</td>
<td>2</td>
<td>5.68 (1.30)</td>
<td>3.75 (0.47)</td>
<td>4.46 (0.40)</td>
<td>-1.93</td>
<td>-1.21</td>
<td>N/A</td>
</tr>
<tr>
<td>014</td>
<td>3</td>
<td>5.05 (0.83)</td>
<td>3.67 (0.40)</td>
<td>5.46 (1.01)</td>
<td>-1.38</td>
<td>0.41</td>
<td>N/A</td>
</tr>
<tr>
<td>015</td>
<td>2</td>
<td>6.75 (0.66)</td>
<td>5.98 (0.62)</td>
<td>3.72 (1.42)</td>
<td>-0.77</td>
<td>-3.03</td>
<td>N/A</td>
</tr>
<tr>
<td>016</td>
<td>1</td>
<td>3.05 (0.39)</td>
<td>4.16 (1.10)</td>
<td>N/A</td>
<td>1.11</td>
<td>N/A</td>
<td>20.09</td>
</tr>
<tr>
<td>017</td>
<td>1</td>
<td>4.10 (0.65)</td>
<td>7.03 (1.87)</td>
<td>3.62 (1.27)</td>
<td>2.93</td>
<td>-0.475</td>
<td>20.09</td>
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<tr>
<td>018</td>
<td>2</td>
<td>4.06 (0.76)</td>
<td>4.06 (1.60)</td>
<td>3.00 (0.99)</td>
<td>0.00</td>
<td>-1.06</td>
<td>20.09</td>
</tr>
<tr>
<td>019</td>
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<td>4.41 (1.88)</td>
<td>2.89 (0.75)</td>
<td>5.95 (2.08)</td>
<td>-1.51</td>
<td>1.09</td>
<td>16.79</td>
</tr>
<tr>
<td>020</td>
<td>1</td>
<td>3.39 (1.06)</td>
<td>4.65 (1.20)</td>
<td>7.23 (4.68)</td>
<td>1.26</td>
<td>3.84</td>
<td>16.79</td>
</tr>
<tr>
<td>021</td>
<td>2</td>
<td>4.60 (1.88)</td>
<td>7.75 (3.70)</td>
<td>5.28 (2.85)</td>
<td>3.16</td>
<td>0.68</td>
<td>16.79</td>
</tr>
</tbody>
</table>

s.d is standard deviation from 3 samples within the same electrophoresis tank, N/A is result /sample not available. Those in **bold** font experienced grade 3/4 toxicities. Those *highlighted* best response was progressive disease.
Figure 5-1. A bar graph to show the cumulative results of the DNA damage measured in PBLs isolated from blood samples before and after exposure to irinotecan in vivo. Results are an average of the median percentage tail DNA across all 21 patients’ samples. P values were calculated using the independent samples t-test compared to baseline.

The ACA was also unable to detect long term irinotecan exposure as illustrated by the observation that there was no difference in background DNA damage levels from patients prior to receiving their first cycle of treatment compared to those due to receive subsequent cycles (Figure 5-2).

Figure 5-2. A bar graph to show the cumulative results of the DNA damage measured in PBLs prior to receiving the first (n=6) or subsequent (n=15) cycles of irinotecan chemotherapy. The p value was calculated using the independent samples t-test.
5.2.2 Correlation of *in vivo* ACA results with clinical data

Seven of these 21 patients would have benefitted from a predictive test of irinotecan effect: 3 required a dose reduction during the first 3 cycles of treatment due to the development of grade 3/4 toxicities, 3 received no clinical benefit with the best response being progressive disease and 1 experienced both severe toxicities and progressive disease (Table 5-1).

The initial hypothesis, that those with toxicities would have greater DNA damage levels was not proven; some of the patients experiencing toxicities had less DNA damage following irinotecan exposure than at baseline whilst others had more. Likewise no trends were apparent with the response data (Figure 5-3).

Following an interim analysis demonstrating these negative results, this *in vivo* part of the clinical study was terminated prematurely.
Figure 5-3. Box and whisker plots to investigate the association of change in DNA damage measured using the ACA, at 1 and 24 hours from baseline, with A) toxicities and B) response to treatment. Results presented are for all 21 trial participants, 2 of whom did not have 24 hour data available. The change in % tail DNA was calculated by subtracting the median % tail DNA at baseline (i.e. the sample taken prior to chemotherapy) from the median % tail DNA at each time point. P values were calculated using the independent samples t-test.

5.3 Conclusion

There was no evidence that irinotecan induced significant DNA damage in PBLs in vivo. The small differences in results within each individual at different time points were most likely just due to intrinsic experimental variability and thus, there were no significant correlations between the laboratory results and the clinical findings. These data did not
support the hypothesis behind this study proposal, that DNA damage in PBLs could predict irinotecan effect.

However, even if significant DNA damage had been detected, this method would not have been a useful predictive tool as the testing was performed after drug exposure. For a clinical test to be successful, it would be important to induce and detect DNA damage ex vivo, prior to exposing the patient to the drug. Therefore, if such ex vivo conditions could be optimised then there would still be merit in conducting this study.
6 Optimisation of method: detecting DNA damage in PBLs treated with irinotecan or SN-38 ex vivo

6.1 Introduction

Results from the in vivo component of the clinical study demonstrated that irinotecan did not induce significant levels of DNA damage that could be detected in PBLs following treatment. When this observation became apparent, following analysis of only a few patients’ samples, recruitment was temporarily suspended and exploratory studies were conducted in the laboratory. These experiments aimed to investigate the negative in vivo study results and also to determine whether conditions could be manipulated to enable irinotecan to induce measurable DNA damage ex vivo. In order to generate a successful biomarker, levels of induced DNA damage would need to be of sufficient magnitude to ensure that inter-individual variations could be determined.

6.2 Results and discussion

Possible explanations to account for the negative results in the in vivo study included that a) storage of the PBLs was sub-optimal, b) the ACA was not detecting irinotecan induced SSBs and c) PBLs were an inappropriate normal tissue surrogate. Mechanistic laboratory investigations were therefore conducted on PBLs obtained from healthy volunteers and on HT-29 cells (an established CRC cell line) to investigate each of these theories in turn and to use information gained to develop the ex vivo method.
6.2.1 Storage of the PBLs

Storage of the PBLs was essential for the *in vivo* study in order to ensure that the samples from all time points could be processed simultaneously. The possibility that the results may have been affected by the freezing of PBLs at -80°C prior to analysis was investigated. Initially, the effect of freezing on the viability of PBLs was assessed by performing the trypan blue exclusion assay on samples obtained from 3 different donors. These results demonstrated than in general one third of PBLs did not survive the freezing process (mean percentage viability on thawing 66.2%, s.d 3.75). To assess if this decline in viability affected the ACA results, DNA damage induced by irradiating PBLs with 4 Gy and then either processing immediately or following frozen storage at -80°C was measured. DNA damage levels were small however a significant radiation response was noted in both samples (p<0.001). The difference in median percentage tail DNA was less for the frozen than for the fresh samples (2.52% Vs 3.74% p=0.001). Possible explanations included either a) a different electrophoresis tank or b) the most damaged cells may have died during the freezing process therefore the ACA result may have reflected lower than actual values.

Overall, these data confirmed that DNA damage could be detected in PBLs following a period of storage in the -80°C freezer despite initial concerns regarding the poor recovery of these samples. The freezing process was unlikely to account for the negative results of the clinical study however it would be best avoided in the *ex vivo* study to ensure good viability and to maximise the DNA damage measured.
6.2.2 Treatment of HT29 cells with irinotecan

In addition to providing evidence that DNA damage in PBLs may correlate with irinotecan effect, the preliminary data supporting this study had also demonstrated that 24 hours of irinotecan exposure could induce a dose response in established CRC cell lines (Smith et al., 2007). Having refuted the first finding, the next step was to confirm that the ACA could detect irinotecan induced DNA damage in a CRC cell line (Figure 6-1).

![Bar chart illustrating the dose response of HT-29 cells treated with irinotecan for 24 hours as measured by the ACA.](image)

**Figure 6-1. A bar chart illustrating the dose response of HT-29 cells treated with irinotecan for 24 hours as measured by the ACA.** Standard deviation was calculated from triplicates within one electrophoresis tank. * indicates the lowest dose that was statistically significant when compared to the 0 µM control with p calculated using Mann Whitney U test.

This result confirmed that the assay setup and irinotecan stock were satisfactory. The next logical step was to see if this dose response could be replicated in PBLs (donated by healthy volunteers) treated with irinotecan ex vivo.
6.2.3 Treatment of PBLs with irinotecan ex vivo

PBLs were isolated and treated with irinotecan (0 – 100 μM) for 24 hours. In contrast to the HT-29 cells, no dose response was demonstrated (Figure 6-2 A). This experiment was repeated with minor modifications using, a different PBL donor, a new stock of irinotecan and higher treatment doses up to 800 μM but again no significant response was noted (Figure 6-2 B).

![Graphs illustrating the dose response of PBLs treated with low and high doses of irinotecan for 24 hours ex vivo measured using the ACA.](image)

**Figure 6-2.** Graphs illustrating the dose response of PBLs treated with A) low and B) high doses of irinotecan for 24 hours *ex vivo* measured using the ACA. Data shown are duplicates from within the same electrophoresis tank for each experiment. The standard error was calculated from 100 cells that were scored per slide. The PBLs were obtained from a different donor and a new stock of irinotecan was used in experiment B, compared to experiment A.
The reported $C_{\text{max}}$ of irinotecan in phase I studies ranges from 2.3 – 13 µg/ml (3.40 – 19.20 µM) (Abigerges et al., 1995) thus both physiological and supra-physiological doses had been investigated and had not produced any response.

### 6.2.4 Treatment of PBLs with SN-38 ex vivo

On the back of the data illustrated in Figure 6-2, it was proposed that the failure of irinotecan to generate an ex vivo dose response could have been due to the inefficiency of PBLs in converting irinotecan to its active metabolite SN-38. The key enzyme to catalyse this conversion is CES2 which is expressed predominantly in the liver but has only been detected at low levels in PBLs and in the blood plasma (Chazal et al., 1996, Guemei et al., 2001) (section 8.2.3).

PBLs were thus treated with SN-38 directly. In early phase clinical studies, the $C_{\text{max}}$ of SN-38 ranged between 0.08 – 0.76 µM (Rivory et al., 1997, Abigerges et al., 1995). No significant dose response was detected to sub clinical, clinical and supra-physiological doses (0.01 - 25 µM) (Figure 6-3).
Figure 6-3. A bar graph illustrating the DNA damage, measured using the ACA, induced in PBLs following 24 hours ex vivo treatment. Error bars represent the standard error that was calculated from 100 cells that were scored per slide.

Taking into account that the in vivo study (during which irinotecan would have circulated through the liver and thus been exposed to CES2) yielded negative results, it was perhaps unsurprising that no dose response was demonstrated with ex vivo exposure to SN-38 either. A shorter duration of drug exposure also failed to elicit a large dose response although there was an indication that some DNA damage was induced following only 1 hour of drug exposure (representative data are shown in Figure 6-8). At this stage it was clear that PBLs in their unaltered form were not a suitable surrogate in which to assess irinotecan / SN-38 effect.

6.2.5 Cell cycle investigations

The next phase of this research was to determine which specific traits of PBLs led to them being unable to acquire DNA damage when exposed to irinotecan or SN-38 both in and ex vivo. The mechanism of action of irinotecan being a topo I inhibitor was thus addressed.
Although topo I is present throughout the cell cycle, its expression increases during cell proliferation (Vanhoef er et al., 2001). In addition, irinotecan is most cytotoxic when the replication fork is advancing in S phase. It was therefore considered that the topo I levels in PBLs, which usually reside in phase G0 of the cell cycle (Richman, 1980) (Figure 6-4 A) were potentially below the level required to induce and detect SSB formation. Additionally, if the cells were not progressing through S phase, the subsequent DSBs would not be formed.

PBLs were therefore stimulated to proliferate. Up to this point blood samples had been collected in vials containing EDTA as an anticoagulant and the PBLs had been cultured in RPMI media containing 10% FCS. As EDTA is known to suppress PBLs’ response to mitogens (Yang and Schultz, 1982), it was important to ensure that blood bottles used contained heparin and not EDTA as the anticoagulant. Quantum 724 complete media for primary lymphocyte culture (QBL) was purchased. This media not only contained a PBL pretested FCS but also the mitogen phytohaemagglutinin (PHA), at a dose adjusted to the mitotic index, antibiotics (penicillin and streptomycin) and L-Glutamine. PBLs were confirmed to cycle using this QBL media (Figure 6-4 B). In addition, the cell cycle analysis of cells cultured in RPMI media had the presence of a sub G1 peak implying that apoptosis was occurring under these suboptimal conditions (Figure 6-4 A).
6.2.6 Treatment of cycling PBLs with irinotecan or SN-38 ex vivo

Following 72 hours in culture in QBL media, the PBLs were treated with irinotecan or SN-38 over a time course (Figure 6-5). These results did successfully demonstrate that the ACA could detect DNA damage in cycling PBLs following exposure to either of these agents ex vivo. The response was maximal following 1 hour of drug exposure and reduced over time. The active metabolite SN-38 produced a greater response than the prodrug irinotecan. At the earlier time points, DNA damage positively correlated with the dose of SN-38 but this was not the case with the later time points.
Figure 6-5. DNA damage, measured using the ACA, induced in PBLs cultured in QBL media treated with irinotecan or SN-38 *ex vivo* over a 12 hour time course. Error bars represent the standard deviation from duplicates within the same tank. Experiments were limited to only 36 slides due to the limited capacity of the electrophoresis tanks.

This experiment was repeated, using single doses of irinotecan and SN-38. The dose of irinotecan used was 200 times greater than the SN-38 dose (100 μM versus 0.5 μM) to account for the difference in potency. These data confirmed that a) the response was greater with SN-38 than with irinotecan and b) damage was maximal following 1 hour of treatment and decreased over time (Figure 6-6). Possible explanations for the decline in DNA damage over time, despite continuous drug exposure, included that a) the sensitive cells (those in S phase) apoptosed over the duration of the treatment and thus only insensitive cells in G₀G₁ were viable when the ACA was performed or that b) the damaged cells had repaired.
Figure 6-6. Repeat of the DNA damage, measured using the ACA, induced in PBLs cultured in QBL media treated with irinotecan or SN-38 \textit{ex vivo} over a 12 hour time course. Error bars represent the standard deviation from duplicates within the same tank.

Having confirmed the optimal treatment duration, more detailed dose response experiments were then conducted using a 1 hour drug treatment time (Figure 6-7).

Figure 6-7. The dose response of PBLs cultured in QBL media treated with A) irinotecan or B) SN-38 \textit{ex vivo} for 1 hour. Standard deviation was calculated from triplicates within one electrophoresis tank. * indicates the lowest dose that was statistically significant when compared to the 0 µM dose calculated using the Mann Whitney U test.
The importance of stimulating PBLs to maximise the DNA damage induced was confirmed by performing a dose response on PBLs from a single donor that were cultured either in the presence (QBL media) or absence (RPMI media) of PHA. These cells were obtained, treated and processed simultaneously within the same electrophoresis tank. The dose response was significantly greater in the cycling PBLs (Figure 6-8).

**Figure 6-8.** A graph to demonstrate the dose response of PBLs cultured in the presence or absence of a mitogen, treated with SN-38 ex vivo as measured using the ACA. Standard deviation was calculated from triplicates within one electrophoresis tank. Those labeled with * were statistically significant when the stimulated were compared to the unstimulated PBLs at the equal dose calculated using the Mann Whitney U test.

As topo I inhibitors exert their cytotoxicity during the advancement of the replication fork whereby SSBs are transformed into DSBs, the effect of PBL proliferation on increasing DSB induction was subsequently confirmed by using flow cytometric measurement of γ-H2AX (Figure 6-9).
Figure 6-9. A graph to demonstrate the dose response of PBLs cultured in the presence or absence of a mitogen, treated with SN-38 for 1 hour ex vivo as measured using flow cytometric detection of γ-H2AX. The results and standard deviation presented were calculated from 2 independent experiments.

6.2.7 Development of the protocol for the *ex vivo* study

The data from these preliminary, exploratory studies were subsequently used to design the protocol for an *ex vivo* component of the clinical study.

The following assay conditions were chosen in order to optimise the levels of DNA damage measured:

- Freezing of PBLs was avoided
- PBLs were cultured in QBL media for 72 hours prior to drug exposure
- Treatment was with SN-38 rather than irinotecan
- A dose response following 1 hour of SN-38 exposure (0 – 5 μM) was performed
- A time course to 5 μM SN-38 treatment over 0 – 10 hours was performed
A potential weakness in this protocol was that by treating the PBLs with SN-38, the opportunity to detect any inter-individual variation due to differences in the metabolism of the irinotecan prodrug was lost. However, as the majority of irinotecan toxicities are thought to be due to the slow glucuronidation of SN-38 (section 1.6.5), it was decided that the higher DNA damage levels induced using SN-38 would be more informative and more likely to detect inter-individual differences than the low levels detected using irinotecan exposure thus outweighing the potential disadvantage of not accounting for variation in irinotecan hydrolysis.

For the dose response experiments it was desirable to study a wide range of doses including a physiological dose. The time course component to the trial was necessary as the decrease in DNA damage over time remained unexplained. When designing this experiment, the logical dose to use would have been a physiological dose (~0.1 μM). However, data from healthy volunteers demonstrated that DNA damage induced using these lower doses were relatively small in comparison to the higher doses (Figure 6-7 and Figure 6-8) although the trait of DNA damage reducing with time of drug exposure was again apparent (Figure 6-10). The decision was therefore taken to use a 5 μM dose of SN-38 that induced a large amount of DNA damage so the relative decrease over time and thus inter-individual differences could easily be observed. Ten hours was chosen as the maximum time point as by this time, damage measured using the ACA was almost back to baseline levels (Figure 6-5 and Figure 6-6). This allowed the additional benefit of being able to make all slides in a single day which could therefore be processed in the same
electrophoresis tank, thus reducing one source of potential intra-individual variation (Zainol et al., 2009).

Figure 6-10. Bar chart to show DNA damage, measured using the ACA, induced in PBLs treated with 0, 0.1 and 5 μM SN-38 ex vivo for 1 and 4 hours following 72 hours culture in QBL media. Error bars represent the standard deviation calculated from triplicates within the same tank.

A DMSO only control sample was processed at each time point for the first 2 patients investigated using this method in the clinical study, with the consequent disadvantage that each patient’s samples had to be analysed over 2 electrophoresis tanks due to the number of slides required. An analysis of these 2 patients and those of 2 healthy volunteers demonstrated no significant difference in baseline damage over time (Figure 6-11) therefore, only a single 1 hour control was performed with subsequent samples thus enabling all slides to be processed in the same tank. It was not possible to study more than one dose over the time course due to a) the larger number of cells that would be required and b) the limiting capacity of the electrophoresis tank.
6.3 Conclusion

Minimal DNA damage was induced in unmanipulated PBLs treated with irinotecan or SN-38 ex vivo. This was explained by demonstrating that these cells do not proliferate and usually reside in phase G0G1 of the cell cycle. DNA damage was induced and detected using both the ACA and by measurement of \( \gamma \)-H2AX if PBLs were stimulated to cycle and thus presumably increasing the expression of topo I (to which SN-38 binds) prior to drug exposure. These preliminary, explorative data were used to develop the method to proceed with the ex vivo component of the clinical study.

Figure 6-11. Baseline DNA damage measured using the ACA in control PBLs over a 10 hour time course for 4 individuals. Standard deviation was calculated for triplicate samples within each electrophoresis tank.
7 The *ex vivo* study results: correlating DNA damage induced in PBLs with toxicities and response to irinotecan treatment

7.1 Introduction

This chapter presents the results that were obtained when the method developed to induce DNA damage in PBLs *ex vivo* (chapter 6) was applied to samples acquired from patients prior to receiving irinotecan based chemotherapy. The aims were to determine whether inter-individual differences in the magnitude and duration of these DNA damage levels existed and more importantly, if such variations were present, whether significant correlations with the clinical effect could be sought. This could then provide evidence as to whether SN-38 induced DNA damage in PBLs *ex vivo* is a predictive biomarker of patients’ toxicities and/or response to irinotecan treatment.

7.2 Results and discussion

Dose response and time course experiments were performed on PBLs obtained from 40 of the 42 trial participants (the first 2 patients recruited only had the *in vivo* assay performed). The results demonstrated a wide range of inter-individual variation in the level of DNA damage induced and detected; correlations of both raw and corrected laboratory results with clinical response and toxicity data were investigated as described below.

7.2.1 Dose response

Raw data are summarised in Table 7-1.
Table 7-1. *Ex vivo* study results: Raw data demonstrating the dose response of PBLs treated with SN-38 for 1 hour assessed using the ACA

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Treatment regimen &amp; cycle sampled</th>
<th>Median % tail DNA (s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM SN-38</td>
<td>0.01 μM SN-38</td>
</tr>
<tr>
<td>003</td>
<td>FOLFIRI 11</td>
<td>3.71 (0.63)</td>
</tr>
<tr>
<td>004</td>
<td>FOLFIRI 2</td>
<td>6.98 (1.45)</td>
</tr>
<tr>
<td>005</td>
<td>FOLFIRI 2</td>
<td>4.13 (1.28)</td>
</tr>
<tr>
<td>006</td>
<td>FOLFIRI 2</td>
<td>6.6 (2.12)</td>
</tr>
<tr>
<td>007</td>
<td>FOLFIRI 10</td>
<td>4.06 (1.50)</td>
</tr>
<tr>
<td>008</td>
<td>FOLFIRI 6</td>
<td>5.11 (0.19)</td>
</tr>
<tr>
<td>009</td>
<td>FOLFIRI 1</td>
<td>4.30 (0.29)</td>
</tr>
<tr>
<td>010</td>
<td>FOLFIRI 3</td>
<td>6.24 (1.34)</td>
</tr>
<tr>
<td>011</td>
<td>FOLFIRI 1</td>
<td>6.33 (1.31)</td>
</tr>
<tr>
<td>012</td>
<td>FOLFIRI 4</td>
<td>7.80 (1.32)</td>
</tr>
<tr>
<td>013</td>
<td>FOLFIRI 2</td>
<td>5.39 (1.54)</td>
</tr>
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<td>014</td>
<td>FOLFIRI 3</td>
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<td>016</td>
<td>FOLFIRI 1</td>
<td>6.47 (1.12)</td>
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<td>Patient ID</td>
<td>Treatment regimen &amp; cycle sampled</td>
<td>0 μM SN-38</td>
</tr>
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<td>-------------</td>
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<td>5.51</td>
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<td>FOLFIRI 1</td>
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</tr>
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<td>023*28</td>
<td>FOLFERA3</td>
<td>3.04</td>
</tr>
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<td>4.11</td>
</tr>
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<td>Patient ID</td>
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<td>Median % tail DNA (s.d.)</td>
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<td>-----------------------------------</td>
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<td>Cap/iri 1</td>
<td>3.46 (0.24)</td>
</tr>
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<td>FOLFIRI 1</td>
<td>4.04 (0.82)</td>
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<td>3.81 (0.91)</td>
</tr>
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<td>FOLFIRI/avastin 3</td>
<td>4.49 (0.83)</td>
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<td>FOLFERA 1</td>
<td>3.81 (0.52)</td>
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<td>041</td>
<td>FOLFERA 1</td>
<td>5.48 (1.21)</td>
</tr>
<tr>
<td>042 28</td>
<td>FOLFIRI 1</td>
<td>4.94 (1.81)</td>
</tr>
</tbody>
</table>

s.d is standard deviation from 3 samples within the same electrophoresis tank, N/A is result/sample not available. Those in **bold** font experienced grade 3/4 toxicities (diarrhoea or neutropenia). Those highlighted had a best response of progressive disease.

The standard deviations presented were calculated from triplicate samples processed within the same electrophoresis tank. These values were particularly large in both the PBLs and HT29 cell controls in sample 005 illustrating that the ACA may be prone to experimental variability; the most likely explanation was that the electrophoresis voltage was not even throughout the tank. This confirmed the importance of processing triplicate samples distributed throughout the electrophoresis tank to minimise such variability.

A dose response was detected in all patients as illustrated by an initial increase in DNA damage with increasing SN-38 dose followed by a plateau at the higher doses when the response became saturated. There were inter-individual variations in the magnitude, steepness and level of plateau of these dose response curves; representative graphs are shown in Figure 7-1. These parameters were all investigated for associations with the clinical data.

![Graph showing dose response curves for PBLs treated with SN-38](image)

**Figure 7-1. Examples of the dose response, measured using the ACA, of PBLs treated with SN-38 for 1 hour ex-vivo.** Individual A had high levels of DNA damage, B had intermediate levels with an early plateau at 1 μM sn38, and C had low levels of DNA damage and a low gradient of the initial dose response (0-0.5 μM).
7.2.1.1 Level of plateau of dose response curve

The absolute maximum DNA damage measured in samples from each individual was detected at the highest (5 μM) treatment dose of SN-38 used in 27 (68%) of the patients (i.e. there was no plateau evident at lower doses). This was in contrast to the remainder, who had maximum damage detected following exposure to lower doses and therefore demonstrated plateauing of the dose response curve (6 at 2.5 μM, 6 at 1 μM and 1 at 0.5 μM). There was no significant correlation of the dose of ACA response saturation with UGT1A1 status nor with toxicities to treatment; however there was a suggestion of an association with clinical response, as illustrated by the fact that none of the patients with progressive disease (PD) exhibited a plateau at doses lower than 5 μM however this finding did not achieve statistical significance (p= 0.075) (Table 7-2).

Table 7-2. The dose of assay saturation in all trial participants and those grouped according to UGT1A1 status, toxicities and response to irinotecan treatment

<table>
<thead>
<tr>
<th>Dose of ACA saturation (μM SN-38)</th>
<th>All patients</th>
<th>Toxicity groups</th>
<th>Response groups</th>
<th>UGT1A1 *28 groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤ Grade 2</td>
<td>Grade 3/4</td>
<td>Clinical benefit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>toxicities</td>
<td>toxicities</td>
<td>(PR/SD)</td>
</tr>
<tr>
<td>0.5</td>
<td>1 (2%)</td>
<td>1 (3%)</td>
<td>0</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>1</td>
<td>6 (15%)</td>
<td>5 (17%)</td>
<td>1 (10%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td>2.5</td>
<td>6 (15%)</td>
<td>4 (13%)</td>
<td>2 (20%)</td>
<td>5 (18%)</td>
</tr>
<tr>
<td>5</td>
<td>27 (68%)</td>
<td>20 (67%)</td>
<td>7 (70%)</td>
<td>16 (57%)</td>
</tr>
</tbody>
</table>

NB/ T, R and U represent p values calculated using the Chi-Squared test for trend assessing the association of toxicity, response and UGT1A1*28 status respectively with the dose at which the ACA DNA damage response plateaued. T= 0.590, R=0.075 and U=0.333

It is plausible that the requirement of a high dose of SN-38 ex vivo to detect a plateau in the laboratory response may be indicative of resistance to treatment in the clinic and with
increased patient numbers this finding may have reached significance. It is also noteworthy that although this test had 100% sensitivity to detect patients with PD, its positive predictive value (PPV) was poor (27%), with 73% of those whose assay results saturated at 5 μM SN-38 deriving clinical benefit. Conversely, the PPV of the DNA damage plateauing at doses ≤2.5 μM predicting clinical benefit was 100%, however the sensitivity was only 43%. The Investigation of more doses between 2.5 – 5 μM would be required to ascertain the robustness of this observation.

7.2.1.2 Magnitude of raw DNA damage

The maximum DNA damage detected was correlated with clinical outcome and genotype, however no associations were found (Figure 7-2). Similarly, the percentage tail DNA detected at sub-physiological, physiological and supra-physiological doses was investigated but once again, when patients were classified according to either UGT1A1*28 status, toxicities or response to chemotherapy, no significant differences in DNA damage between these groups were detected (Figure 7-3). Additionally, there were no significant associations of raw DNA damage at any dose with progression free survival (PFS) or overall survival (OS) (Figure 7-4 and Figure 7-5).
Figure 7-2. Bar graphs and box and whisker plots to show the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with maximum raw DNA damage, measured using the ACA, induced in PBLs treated with 0.01 - 5 μM SN-38 for 1 hour ex vivo. Bar graphs demonstrate the rank order of DNA damage from all individual patients. Box and whisker plots compare patients grouped according to response, toxicity & UGT1A1*28 status. P values were calculated using the independent samples t-test.
Figure 7-3. Bar graphs and box and whisker plots to show the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with raw DNA damage, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 1 hour at doses of A)0.01, B)0.1, C)0.5, D)1.0 and E)2.5 μM. Bar graphs demonstrate the rank order of DNA damage from all individual patients. Box and whisker plots compare patients grouped according to response, toxicity & UGT1A1*28 status. P values were calculated using the independent samples t-test.
Figure 7-3 continued

B) 0.1 μM SN-38

Rank order

Toxicities

Median % tail DNA

Response

UGT1A1*28

Clinical benefit

Progressive disease

Tolerated

Toxicities

p=0.412

p=0.764

p=0.388
C) 0.5 μM SN-38

Figure 7-3 continued
D) 1.0 μM SN-38

Figure 7-3 continued
Figure 7-3 continued
Figure 7-4. Kaplan-Meier plots demonstrating the PFS for patients grouped according to the level of raw DNA damage, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 1 hour at doses of A)0.01, B)0.1, C)0.5, D)1.0 and E)2.5 μM. Figure 7-4 F represents the PFS for patients grouped according to the maximum DNA damage induced across all doses. P values were calculated using the log rank test.
Figure 7-5. Kaplan-Meier plots demonstrating the OS for patients grouped according to the level of raw DNA damage, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 1 hour at doses of A)0.01, B)0.1, C)0.5, D)1.0 and E)2.5 μM. Figure 7-5 F represents the OS for patients grouped according to the maximum DNA damage induced across all doses. P values were calculated using the log rank test.
7.2.1.3 Gradient of the dose response curve

One possible explanation for the lack of associations with the clinical data was assay variability and experimental error. The gradient of the dose response curve between 0 and 0.5 μM was therefore calculated with the aim of minimising error by assessing the average DNA damage over 4 data points. One would hypothesise that a steep gradient would be indicative of a high sensitivity to SN-38 and may thus predict toxicity and response or be associated with the presence of UGT1A1*28*28. However, even a patient with grade 3/4 toxicities had a negative initial gradient and once again, no significant associations with the clinical and survival data were detected (Figure 7-6 and Figure 7-7).
Figure 7-6. Bar graphs and box and whisker plots to show the associations of UGT1A*28 status, response & toxicities to irinotecan chemotherapy with the gradient of DNA damage, measured using the ACA, induced in PBLs treated with SN-38 ex vivo at doses of 0 - 0.5 μM for 1 hour. Bar graphs demonstrate the rank order of gradient from all individual patients. Box and whisker plots compare patients grouped according to response, toxicity & UGT1A*28 status. P values were calculated using the independent samples t-test.
Figure 7-7. Kaplan-Meier plots demonstrating the A) PFS and B) OS for patients grouped according to the gradient of DNA damage, induced in PBLs treated with SN-38 ex vivo at doses of 0 - 0.5 μM for 1 hour. P values were calculated using the log rank test.

7.2.1.4 Magnitude of DNA damage when corrected using HT29 cell controls

All of the samples from each participant were processed in a single electrophoresis tank along with three HT-29 cell controls: a negative control treated with DMSO only to assess background damage, and two positive controls treated with either 1 μM SN-38 or 10 Gy irradiation to assess the potency of the SN-38 and the consistency between the electrophoresis runs. In a further attempt to account for possible experimental variation, the raw results were corrected using data obtained from these controls as described below.

The irradiated and SN-38 treated controls did demonstrate that there was variability between each experiment (Table 7-1) however the DNA damage in the irradiated cells was
more consistent than in those treated with SN-38 (co-efficient of variation 0.25 versus 0.54). The more consistent irradiated controls were therefore used to correct for differences between electrophoresis tanks.

There was no evidence that the SN-38 controls were less damaged over time implying that the differences in results were not due to degradation of the SN-38 in storage. However, these results were of concern as they inferred that the DNA damage induced by SN-38 in vitro may be influenced by several factors (passage number, age of media etc) and thus was not accurately reproducible. Clearly, an essential requirement of a successful biomarker is that the assay not influenced by external factors and subject to high levels of error.

To calculate the correction factor (cf) for each experiment, the difference in the percentage tail DNA between the negative and irradiated controls were calculated by subtraction and then the following formula was applied:

\[ cf = A / M \]

Where cf is the correction factor, A is the average difference in percentage tail DNA across all samples and M is the difference in percentage tail DNA for that individual experiment.

The results from each experiment were then multiplied by the c.f (Table 7-3). Data were available from 37 patients (Figure 7-8).
Figure 7-8. Box and whisker plots investigating the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with DNA damage adjusted using the irradiated control correction factor, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 1 hour at doses of A)0.01, B)0.1, C)0.5, D)1.0 and E)2.5 μM. Figure 7-8 F represents the maximum corrected DNA damage detected across all doses. P values were calculated using the independent samples t-test.
Figure 7-8 continued.
Contrary to the proposed hypothesis, there was no association of DNA damage with toxicities to treatment. There was also no effect of the presence of homozygosity for UGT1A1*28 with DNA damage levels detected. Conversely, in keeping with the original hypothesis, DNA damage was generally lower in those with PD but this did not reach statistical significance. A p value of 0.066 was obtained for the corrected data for the 0.5 μM SN-38 dose and this result may have reached significance with increased patient numbers, however the mean difference in DNA damage between those with toxicities and those without was only 3.05% therefore, even if significant, this finding would be unlikely to transpire into a predictive test because the ACA is not sensitive enough to reproducibly detect such a small difference. This fact was illustrated by the differences between the irradiated controls (Table 7-1). Even so, this result did provide evidence that a more accurate assessment of DNA damage may potentially predict those who will not benefit from treatment.

When analysing survival data, there were no significant correlations between DNA damage and PFS or OS (Figure 7-9 and Figure 7-10).
Figure 7-9. Kaplan-Meier plots demonstrating the PFS for patients grouped according to the level of DNA damage, adjusted using the irradiated control correction factor, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 1 hour at doses of A) 0.01, B) 0.1, C) 0.5, D) 1.0 and E) 2.5 μM. Figure 7-9 F represents the PFS for patients grouped according to the corrected maximum DNA damage induced across all doses. P values were calculated using the log rank test.
Figure 7-10. Kaplan-Meier plots demonstrating the OS for patients grouped according to the level of DNA damage, measured using the ACA, adjusted using the irradiated control correction factor, induced in PBLs treated with SN-38 ex vivo at doses of A) 0.01, B) 0.1, C) 0.5, D) 1.0 and E) 2.5 μM. Figure 7-10 F represents the OS for patients grouped according to the corrected maximum DNA damage induced across all doses. P values were calculated using the log rank test.
7.2.2 Time course

All of the results above have only studied DNA damage following 1 hour of exposure to SN-38. Clearly in vivo, depending on each patient’s individual metabolism, exposure to SN-38 may last for longer than this. Studies of PBLs from healthy volunteers had previously shown that the initial DNA damage measured at 1 hour decreased with time of SN-38 exposure (section 6.2.6). ACA time course experiments were therefore also conducted to investigate variations in DNA damage over 10 hours. As with the dose response experiments above, inter-individual differences in results were present (Table 7-3). Representative time courses are illustrated in Figure 7-11.

![Figure 7-11. Examples of the DNA damage, measured using the ACA, of PBLs treated with 5 μM SN-38 ex-vivo over a 10 hour time course. The majority of patients (n=37) demonstrated profiles similar to individuals A and B with DNA damage being maximal at 1 hour and reducing over time. Two patients, one of which experienced severe toxicities had profiles represented by line C with maximum damage occurring at 4 hours.](image-url)
Table 7-3. *Ex vivo* study results: The raw data to show the DNA damage measured using the ACA, induced in PBLs treated with 5 μM SN-38 over a 10 hour time course.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Total number of treatment cycles received (cycles at full dose)</th>
<th>Median % tail DNA (s.d.)</th>
<th>% decrease in % tail DNA from 1 hour</th>
<th>Correction factor calculated from irradiated controls</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 μM SN-38 1 hour</td>
<td>5 μM SN-38 1 hour</td>
<td>5 μM SN-38 4 hours</td>
</tr>
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<td>003</td>
<td>12 (3)</td>
<td>3.16 (0.59)</td>
<td>18.55 (2.26)</td>
<td>7.31 (1.65)</td>
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<td>004</td>
<td>12 (12)</td>
<td>4.95 (1.51)</td>
<td>18.14 (4.20)</td>
<td>7.54 (0.53)</td>
</tr>
<tr>
<td>005</td>
<td>11 (11)</td>
<td>4.13 (1.28)</td>
<td>10.94 (2.02)</td>
<td>9.94 (3.10)</td>
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<td>12 (12)</td>
<td>6.6 (2.12)</td>
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</tr>
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<td>4.06 (1.50)</td>
<td>25.25 (0.53)</td>
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</tr>
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<td>25.45 (3.26)</td>
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</tr>
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<td>20.23 (5.35)</td>
<td>18.39 (4.62)</td>
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<td>23.06 (1.62)</td>
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<td>21.74 (1.97)</td>
<td>17.88 (3.52)</td>
</tr>
<tr>
<td>Patient ID</td>
<td>Total number of treatment cycles received (cycles at full dose)</td>
<td>Median % tail DNA (s.d.)</td>
<td>% decrease in % tail DNA from 1 hour</td>
<td>Correction factor calculated from irradiated controls</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------</td>
<td>--------------------------</td>
<td>-------------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>0 μM SN-38 1 hour</td>
<td>5 μM SN-38 1 hour</td>
<td>5 μM SN-38 4 hours</td>
<td>5 μM SN-38 10 hours</td>
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<tr>
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<td>12 (6)</td>
<td>6.70 (0.65)</td>
<td>13.06 (2.02)</td>
<td>16.05 (2.90)</td>
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<td>6.47 (1.12)</td>
<td>18.49 (0.89)</td>
<td>15.04 (4.28)</td>
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<td>5.51 (2.65)</td>
<td>31.46 (8.89)</td>
<td>15.16 (0.89)</td>
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<td>9.29 (1.59)</td>
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<td>26.79 (5.53)</td>
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<td>20.76 (0.88)</td>
<td>9.97 (2.04)</td>
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<td>3.67 (0.52)</td>
<td>27.57 (3.72)</td>
<td>11.06 (2.94)</td>
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<td>3.92 (1.90)</td>
<td>12.64 (1.93)</td>
<td>7.21 (0.78)</td>
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<td>3.04 (0.82)</td>
<td>15.47 (1.59)</td>
<td>7.25 (0.98)</td>
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<td>14.07 (0.58)</td>
<td>7.68 (1.17)</td>
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<tr>
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<td>12 (2)</td>
<td>4.30 (0.78)</td>
<td>22.88 (8.88)</td>
<td>10.44 (0.54)</td>
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<td>7.76 (1.62)</td>
</tr>
<tr>
<td>Patient ID</td>
<td>Total number of treatment cycles received (cycles at full dose)</td>
<td>Median % tail DNA (s.d.)</td>
<td>% decrease in % tail DNA from 1 hour</td>
<td>Correction factor calculated from irradiated controls</td>
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<td>Median % tail DNA (s.d.)</td>
<td>% decrease in % tail DNA from 1 hour</td>
<td>Correction factor calculated from irradiated controls</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0 μM SN-38 1 hour</td>
<td>5 μM SN-38 1 hour</td>
<td>5 μM SN-38 4 hours</td>
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<td>4.94 (1.81)</td>
<td>18.89 (3.21)</td>
<td>10.65 (0.56)</td>
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</table>

NB/ s.d is standard deviation from 3 samples within the same electrophoresis tank. N/A is result/sample not available. Those in **bold** font experienced grade 3/4 toxicities (diarrhoea or neutropenia). Those **highlighted** had a best response of progressive disease. *28 = UGT1A1*28 homozygote. # = Treatment still ongoing when data collection was completed.
The mechanism for the decrease in DNA damage over time was as yet unexplained but a possible explanation was that the damage was repaired. Theoretically one could expect those with slow repair to be more likely to experience toxicities. This was supported by 2 out of the 3 patients who had higher damage levels at later time points than at 1 hour experiencing toxicities; patients 012 and 035 experienced severe toxicities due to irinotecan whereas 015 did not (Table 7-3). As with the dose response above, correlations of the raw results with the clinical data were therefore sought but no significant associations were detected (Figure 7-12).

In addition to analysing the raw data, the rate of decrease in DNA damage over time was calculated using the following equation:

\[
\% \text{ decrease in } \% \text{ tail DNA} = \frac{(\text{damage at 1 hour} - \text{baseline damage}) - (\text{damage at timepoint} - \text{baseline damage}) \times 100}{(\text{damage at 1 hour} - \text{baseline damage})}
\]

The advantage of calculating the \% decrease in \% tail DNA was that this could correct for differences between electrophoresis tanks (Figure 7-13).
Figure 7-12. Bar graphs and box and whisker plots to show the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with the DNA damage measured using the ACA, induced in PBLs treated with 5 μM SN-38 ex vivo for A) 4 hours and B) 10 hours. Bar graphs demonstrate the rank order of DNA damage from all individual patients. Box and whisker plots compare patients grouped according to response, toxicity & UGT1A1*28 status. P values were calculated using the independent samples t-test.
Figure 7-12 continued.
Figure 7-13. Box and whisker plots to show the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with the % decrease in % tail DNA damage compared to 1 hour’s drug exposure measured using the ACA, induced in PBLs treated with 5 μM SN-38 for A) 4 hours and B) 10 hours. P values were calculated using the independent samples t-test.

In the absence of any correlations of the raw time course results with the clinical data, the correction factor calculated in section 7.2.1.4 was also applied. Again, no significant associations of response, toxicities and UGT1A1*28 status with the corrected results were detected (Figure 7-14).
Figure 7-14. Box and whisker plots to investigate the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with the DNA damage measured using the ACA, adjusted using the irradiated control correction factor, induced in PBLs treated with 5 μM SN-38 for A) 4 hours and B) 10 hours. P values were calculated using the independent samples t-test.

Finally, correlations of the time course data with survival were investigated. Again, there were no significant associations between raw DNA damage levels at 4 and 10 hours of drug exposure with survival. The corrected data was significant at 10 hours (Figure 7-15) but this finding does need to be interpreted with caution as patient numbers were relatively small (n=31). There were no associations between DNA damage and overall survival (Figure 7-16).
Figure 7-15. Kaplan-Meier plots demonstrating the PFS for patients grouped according to A) the level of DNA damage, B) the % decrease in % tail DNA and C) DNA damage adjusted using the irradiated control correction factor, measured using the ACA, induced in PBLs treated with 5 μM SN-38 ex vivo for 4 and 10 hours. P values were calculated using the log rank test. *= statistically significant.
Figure 7-16. Kaplan-Meier plots demonstrating the OS for patients grouped according to A) the level of DNA damage, B) the % decrease in % tail DNA and C) DNA damage adjusted using the irradiated control correction factor, measured using the ACA, induced in PBLs treated with SN-38 ex vivo for 4 and 10 hours. P values were calculated using the log rank test.
7.3 Analysis of selected cases

Up to this point all available data was been analysed. No significant trends with toxicity, response or UGT1A1 status were demonstrated. There was a significant finding that increased corrected DNA damage did correlate with improved PFS but this did not translate to an OS benefit. To assess whether these trends could be strengthened if the assay variability was less, once again the irradiated HT-29 cells control results were reviewed. The mean (13.56) and standard deviation (4.34) in the difference in the percentage tail DNA between the negative and irradiated controls was used to select only those samples for which the individual percentage tail DNA difference lay within 1 standard deviation of the mean (9.22 – 17.90). Thus on this basis, 22 patients were selected to have similar assay efficacy. Clearly this analysis was limited due to smaller patient numbers but, within this selected group, 6 had severe toxicities, 4 had progressive disease and 2 were UGT1A1*28 homozygotes. A correction factor from these selected cases was calculated as detailed in section 7.2.1.4. Data that were closest to significance previously (namely 0.01 and 0.5 μM for the dose response) and 4 and 10 hours the time course were re-analysed in this subset of patients (Figure 7-17 and Figure 7-18). Survival was also calculated from this selected corrected data set (Figure 7-19 and Figure 7-20)
Figure 7-17. Box and whisker plots to investigate the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with the DNA damage of cases selected according to irradiated control being within 1 standard deviation of the mean for all controls, measured using the ACA, adjusted using the selected irradiated control correction factor, induced in PBLs treated ex vivo with A) 0.01 and B) 0.5 μM SN-38 for 1 hour. P values were calculated using the independent samples t-test.
Figure 7-18. Box and whisker plots to investigate the associations of UGT1A1*28 status, response & toxicities to irinotecan chemotherapy with the DNA damage of cases selected according to irradiated control being within 1 standard deviation of the mean for all controls, measured using the ACA, adjusted using the selected irradiated control correction factor, induced in PBLs treated with 5 μM SN-38 ex vivo for A) 4 hours and B) 10 hours. P values were calculated using the independent samples t-test.
Figure 7-19. Kaplan-Meier plots demonstrating the PFS for patients selected according to irradiated control being within 1 standard deviation of the mean grouped according to level of DNA damage adjusted using the selected irradiated control correction factor, measured using the ACA, induced in PBLs treated \textit{ex vivo} with: A) 0.01 μM SN-38 for 1 hour, B) 0.5 μM SN-38 for 1 hour, C) 5 μM SN-38 for 4 hours and D) 5 μM SN-38 for 10 hours \textit{ex vivo}. P values were calculated using the log rank test.
**Figure 7-20** Kaplan-Meier plots demonstrating the OS for patients selected according to irradiated control being within 1 standard deviation of the mean grouped according to level of DNA damage adjusted using the selected irradiated control correction factor, measured using the ACA, induced in PBLs treated *ex vivo* with: A) 0.01 μM SN-38 for 1 hour, B) 0.5 μM SN-38 for 1 hour, C) 5 μM SN-38 for 4 hours and D) 5 μM SN-38 for 10 hours *ex vivo*. P values were calculated using the log rank test.

In keeping with the earlier analysis, no significant associations with UGT1A1*28 status, toxicities and response to treatment were demonstrated. This limited data set did
demonstrate a significant increase in PFS time in those with high levels of DNA damage at 4 hours but this result needs to be interpreted with caution as this analysis only accounted for 40% of the total patients recruited.

### 7.4 Conclusion

There was a wide inter-individual variation in the DNA damage levels, induced *ex vivo* in PBLs obtained from patients prior to receiving irinotecan based chemotherapy, measured using the ACA. There were no significant associations with the dose response (0 – 5 μM SN-38 over 1 hour) or the time course (5 μM SN-38 over 1 – 10 hours) results with the response or toxicities to irinotecan treatment thus the original hypotheses that: a) those who experienced toxicities would have relatively high levels of DNA damage and b) those with progressive disease would demonstrate low levels of DNA damage, were not confirmed.

Theoretically, if DNA damage was a biomarker of irinotecan effect, one would have expected those believed to be slow metabolisers of SN-38 (namely those known to be UGT1A1*28 homozygotes) to have higher levels of DNA damage persisting for longer. This was also not confirmed although it is noteworthy that only 1 of the patients homozygous for this polymorphism actually experienced toxicities to treatment (section 4.7) suggesting that there were other more dominant factors affecting SN-38 metabolism in these individuals.

There was however, some weak evidence that DNA damage may be a biomarker to predict irinotecan effect. Firstly, it was noted that all patients experiencing PD had saturation of the dose response at the maximum dose of SN-38 used whereas the
response saturated at lower doses in some of those with clinical benefit. The main limitation of this finding was that its positive predictive value was poor.

It was also noteworthy that when analyzing the corrected and selected data, the PFS was significantly longer in those having higher DNA damage levels at 10 and 4 hours respectively. Due to the small patient numbers, and correction factors required these results cannot be deemed robust and would require further studies to validate them.

The potential use of this finding in the clinical setting was also limited as it did not translate into an increase in OS in this patient group (although this could be explained by several of the participants crossing over to third line treatment). The survival data was not entirely comparable with that in the published literature; the median PFS in this study was slightly higher than would be expected (6.6 versus 2.5 – 6.2 months), whereas the OS was only comparable or lower than that previously reported (10 months versus 9.5 – 15.4 months) (section 4.6) but once again, this can be attributed to the relatively small patient numbers recruited.

Experiments to further investigate the mechanism of decrease in DNA damage over time were required to assess whether DNA damage at the later time points was a biologically plausible biomarker.
8 Mechanistic study results: investigating inter-individual variations in DNA damage detected in PBLs measured using the ACA following ex-vivo SN-38 exposure.

8.1 Introduction

Analysis of the clinical study data did not convincingly support the hypothesis that DNA damage measured in PBLs is a biomarker of irinotecan effect, even though wide inter-individual variations in the results were detected (chapter 7). Possible explanations to explain the limited correlations of the laboratory with the clinical data included: a) DNA damage was truly not a predictive biomarker of irinotecan effect, b) the method used to stimulate PBLs and then measure DNA damage with the ACA was not robust and reproducible enough to allow subtle differences between individuals to be accurately detected and c) PBLs were an inappropriate surrogate in which to study irinotecan effect.

Mechanistic studies were therefore conducted in parallel to the clinical study in order to investigate the reasons behind the inter-individual differences in results. If these could be understood they may provide further evidence to accept or reject the project hypothesis that drug induced DNA damage predicts irinotecan effect. Alternatively, understanding of the variation in results may provide information to enable the assay to be refined and thus be more accurately predictive.
8.2 Results and discussion

8.2.1 Investigating inter-individual variation in the magnitude of DNA damage detected

The ACA is prone to minor variations between experiments due to several factors including (Zainol et al., 2009):

- Error during in vitro or ex vivo cell treatments
- Error ensuring homogeneity of the agarose layers
- Buffer variations
- Variations in background damage e.g. light or temperature changes
- Timing inaccuracies e.g. time taken to obtain the correct electrophoresis voltage
- Error ensuring electrical field homogeneity in the electrophoresis tanks

The reproducibility of the assay was therefore subject to further analysis and investigations.

8.2.1.1 Analysis of inter-experimental variation in the HT29 cell controls

As discussed in section 7.2.1.4, inter-experimental variation was noted in the HT29 cell controls with the irradiated cells yielding more reproducible results than those treated with SN-38 (co-efficients of variation 0.25 and 0.54 respectively). If this variation was solely due to experimental error of the ACA, then one would expect the DNA damage of these controls to correlate with each other (i.e. highly damaged irradiated controls would have occurred in the same experiment as highly damaged SN-38 treated ones).

However, this association was insignificant (Figure 8-1 A). None of the irradiated results were identified as outliers, whereas three of the more variable SN-38 results
were so, but the correlation was only relatively weak, albeit statistically significant when these outliers were excluded (Figure 8-1 B).

Figure 8-1. Scatter graphs to assess the correlation between the irradiated and SN-38 treated HT29 cell controls assessed using the ACA in A) all samples analysed and B) with outlying results (n=3) excluded. P values were calculated using the Pearson correlation co-efficient.

These control data therefore illustrated that the SN-38 treatment was more prone to induce experimental variation than irradiation treatment of established cell lines. The irradiated controls were all of the same passage number, treated simultaneously and stored until analysed. This was not possible with the SN-38 controls that were prepared separately prior to each experiment. Any factor that influenced the rate of cell division and thus the expression of topo-I, may theoretically have altered the DNA damage induced by SN-38 treatment. Such factors included: age of media, passage number of cells, confluence of cells and the time since resurrection from liquid nitrogen.
8.2.1.2 Analysis of inter-experimental variation in the clinical PBL samples

As with the established cell lines, experimental variation was also likely to have occurred during the treatment of PBLs ex vivo. The co-efficient of variation of the maximum DNA damage detected in the clinical PBL samples was 0.43 for the raw data and this increased to 0.53 when the correction factor derived from the irradiated controls was applied. If differences were solely due to experimental error, one would expect that applying a correction factor would decrease a co-efficient of variation therefore, the fact that this value increased, indicated that factors other than assay variability were influencing the data. Evidence supporting the influence of external factors was also provided by the presence of only a weak correlation between the irradiated controls and DNA damage in the clinical samples (Figure 8-2).

Figure 8-2. Scatter graphs to assess the correlation between the irradiated HT29 cell controls and the maximum DNA damage detected in the clinical samples measured using the ACA in A) all samples analysed and B) with outlying results (n=1) excluded. P values were calculated using the Pearson correlation co-efficient.
Factors that may have influenced these results included:

- Differences between individuals that may plausibly lead to DNA damage truly being a predictive biomarker of irinotecan effect (e.g. level of topo-I expression and efficacy of pathways involved in SN-38 metabolism).
- Varying efficacy of stimulation; the time taken to isolate PBLs following obtaining the blood samples, the freshness of media and inter-individual variation in response to PHA exposure may all have affected the proportion of cells in S phase following 72 hours in culture. A higher proportion of cells in S phase with higher levels of topo-I expression could theoretically be associated with higher levels of DNA damage.

Therefore, in order to determine whether the results obtained were representative of real inter-individual differences or actually just a manifestation of limitations in the method used, intra-individual variation was studied by performing both the ACA and cell cycle analysis on PBLs extracted from a single healthy volunteer on 3 separate occasions. The standard deviation of DNA damage measured using the ACA in the irradiated controls for these 3 experiments was small (0.58%) giving a co-efficient of variation of just 0.04 therefore no correction factor was applied. The inter-experimental variation was greater at 1 hour for both the 0.1 and 5 μM doses (coefficients of variation 0.41 and 0.42 respectively) compared to 4 hours (0.17 and 0.22) (Figure 8-3). It is noteworthy that the significant correlations in the clinical study were at the later time points, thus demonstrating the possibility that the large co-efficient of variation at 1 hour may have masked any significant associations with the clinical data.
Figure 8-3. Assessing inter-experimental variability in DNA damage induced in PBLs by *ex vivo* exposure to 0.1 and 5 μM SN-38 for 1 and 4 hours measured using the ACA. PBLs were obtained from a single donor on 3 separate occasions. Results presented are the mean and standard deviation from these 3 experiments. Samples were processed alongside the untreated and irradiated HT29 cell controls.

Cell cycle analysis, performed on aliquots of the same PBLs analysed in Figure 8-3 demonstrated that the efficacy of stimulation was reproducible with the mean proportion of cells in S phase following 72 hours in culture being 45.8% (standard deviation 3.58, co-efficient of variation 0.078). There was no significant correlation between the proportion of cells in S Phase (albeit with only small differences between samples) with DNA damaged measured using the ACA following 5 μM *ex vivo* SN-38 exposure ($R^2$-0.4, $p=0.53$).

Overall, these results demonstrated that this method employed, to stimulate PBLs and then measure the magnitude of DNA damage induced following 1 hour’s *ex vivo* exposure to SN-38 using the ACA, may not have been of sufficient sensitivity or reproducibility to conclusively prove or disprove the hypothesis that DNA damage is a predictive biomarker of irinotecan effect.
8.2.2 Investigating inter-individual variation in the time course experiments

In almost all patients, the DNA damage detected in PBLs following ex vivo SN-38 exposure was maximal after 1 hour of treatment and decreased over time. It was necessary to elicit the mechanism for this decline in damage, not only to explain the heterogeneity in results, but also to assess the plausibility of using these differences as predictive biomarkers, in particular as the corrected data had demonstrated a significant association of higher levels of DNA damage at the later time points with improved PFS (section 7.2.2). Mechanisms that were potentially contributing to the decline in DNA damage over time included:

- Conversion of the SN-38 from its lactone to its carboxylate form
- Deactivation of SN-38 by glucuronidation and its removal from the cells by ABC pumps
- Repair of the drug induced damage
- Apoptosis or necrosis of most damaged cells, resulting in only the least damaged cells being available for analysis

In addition, despite continuous drug exposure, it seemed unlikely that new SSBs were being induced at later time points as illustrated by the low DNA damage levels detected. This may in part have been due to deactivation of the drug or conversion to its carboxylate form. Alternatively, it was plausible that cell cycle arrest due to unrepaired damage triggering check point control was occurring, thus reducing the proportion of PBLs in S phase and consequently restricting the formation of new damage.

Each of these mechanisms was considered / investigated in turn.
8.2.2.1 Carboxylate form of SN-38

If, during treatment, the SN-38 was converted from its active (lactone) to its inactive (carboxylate) form by pH dependant hydrolysis (section 1.6.1) the potency of the drug and thus the DNA damage levels detected may have decreased. Whilst this process may have accounted for some of the decline in DNA damage over time, it was deemed unlikely that this would have contributed to the inter-patient variability because stability studies had previously demonstrated that SN-38 lactone concentrations in media disappeared at the same rate regardless of whether cells were present or not, initially declining and reaching equilibrium with the inactive carboxylate form by 4 hours (Cummings et al., 2002).

8.2.2.2 Glucuronidation

Complete deactivation of the SN-38 by glucuronidation was deemed unlikely as the treatment dose used for the time course experiments was supra-physiological (i.e 5 µM versus physiological levels of ~0.1µM). This would therefore be likely to be too high a concentration for normal cells to completely metabolise. Further evidence against glucuronidation was provided in the clinical study whereby those patients anticipated have slow deactivation of SN-38 (namely the UGT1A1*28 homozygotes) did not have significantly higher DNA damage at the later time points (section 7.2.2).

The effect of cell number on the ACA results was also investigated. It was hypothesised that if PBLs were metabolising SN-38, then a sample containing a high cell count would deactivate an equal amount of SN-38 more rapidly than if a smaller number of cells were treated, however this was not shown (Figure 8-4).
Figure 8-4. The effect of PBL number treated on the DNA damage, measured using the ACA, induced by ex vivo exposure to 5 μM SN-38 over a 4 hour time course. Results presented are the mean and standard deviation from PBLs from one healthy donor, analysed in triplicate in a single experiment.

Finally, to assess whether the ACA time course was influenced by the rate of glucuronidation, PBLs were treated with SN-38 ex vivo in the presence and absence of saquinivir – a UGT inhibitor (Zhang et al., 2005). Results demonstrated that addition of the UGT inhibitor had no effect on the DNA damage levels detected (Figure 8-5). This finding should, however, be interpreted with caution as it was not confirmed that saquinivir definitely inhibited UGT ex vivo in this setting. An additional confounding factor was that saquinivir was not specific for UGT and is known to interact with other enzyme systems including CYP450 that are also involved in irinotecan metabolism (Zhang et al., 2005).
Figure 8-5. Mean results from 2 individual experiments, each conducted in triplicate to investigate the effect of adding saquinavir (a UGT inhibitor) on the DNA damage measured in PBLs using the ACA treated with SN-38 \textit{ex vivo} over an 8 hour time course.

Taking these results altogether there was no evidence that PBLs glucuronidated SN-38 \textit{ex vivo}. This raised doubt as to whether PBLs were an appropriate surrogate in which to study irinotecan / SN-38 effect because the rate of glucuronidation is believed to correlate with toxicities to treatment (section 1.6.5). This was further investigated using high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) (section 8.2.3).

8.2.2.3 Repair of the drug induced damage

To repair SN-38 induced DNA damage, firstly the trapped topo-I complex needs to be relieved of the bound drug by proteolysis. This process could in theory occur at different rates between individuals and thus if it occurred relatively efficiently the DNA damage detected at the later time points would be less than if proteolysis was inefficient.
Once the trapped topo-I complex has been removed, DNA repair may then occur (Covey et al., 1989). SSBs are usually repaired within minutes (Caldecott, 2008), however homologous recombination (HR) (the predominant pathway of repair of DSBs that are produced when the replication fork collapses) (section 1.6.3.2) is more complex and time consuming as it involves the broken DNA ends using homologous sequences elsewhere in the genome (sister chromatids, homologous chromosomes, or repeated regions on the same or different chromosomes) to prime repair synthesis (Shrivastav et al., 2008). It was thus proposed that the high levels of initial damage at 1 hour measured using the ACA predominantly represented SSBs, whereas the damage at later time points could be due to persisting DSBs, that occurred less frequently and took longer to repair. Those with inefficient repair would therefore have higher levels of damage at the later time points.

8.2.2.3.1 DSB repair in Chinese Hamster Ovary (CHO) cell lines

To investigate the effect of DSB repair on the ACA time course results, two Chinese hamster ovary (CHO) cell lines namely AA8 (a wild type phenotype expressing proficient DSB repair) and irs1SF (defective in the HR repair gene XRCC3) (Hinz et al., 2003) were treated with SN-38 in vitro. Although the overall magnitude of DNA damage induced was small, the ACA results demonstrated that damage levels reduced over time in AA8 cells but in contrast, in the irs1SF cells, these levels actually increased (Figure 8-6). These findings supported the theory that the drop in ACA damage over time in PBLs may be as a result of repair, and the damage persisting at the later time points may be due to unrepaired DSBs.
To further investigate this theory, the formation of DSBs in these CHO cells was more specifically studied by assessing the formation of γ-H2AX foci following in vitro SN-38 treatment (Figure 8-7).
The AA8 cells had maximum DSBs detected after 4 hours of drug exposure which was in contrast to the maximum damage measured using the ACA (predominantly SSBs) being present after only 1 hour of treatment. This therefore supported the theory that the high initial levels of DNA damage as measured using the ACA in PBLs were predominantly due to the more rapidly repairable SSBs and the persisting damage detected at 4 and 10 hours was due to unrepaired DSBs.

However, results from the irs1SF cells did not provide further support for this theory as although they had higher levels of background DSBs than the AA8 cells, there was no evidence of DSB induction with SN-38 exposure (Figure 8-7). One possible explanation as to why, when exposed to SN-38 in vitro, irs1SF cells displayed SSBs measured by the ACA but did not acquire any detectable DSBs measured by counting γ-H2AX foci was that SN-38 interacted with the cell cycle; irs1SF cells were shown to arrest in G2M (Figure 8-8) thus the replication fork may not have advanced to reveal the DSBs. Alternatively, it is plausible that irs1SF cells do not actually form γ-H2AX foci as a result of their repair deficiency. Therefore, rather than further investigating the CHO cell lines, it was deemed more prudent to assess DSBs in PBLs directly.
8.2.2.3.2 DSB repair in PBLs

Clinical samples were treated with 5 μM SN-38 for up to 10 hours. Images revealed that following treatment, the uptake of the Alexa Fluor™ stain was so great that it was not possible to count individual foci. A decision was therefore made to calculate area stained per cell instead of individual foci (Figure 8-9).

Figure 8-8. Raw data and bar charts to demonstrate the effect on treatment with 5 μM SN-38 for 24 hours on the cell cycle in A) AA8 and B) irs1SF cells. Only the raw data from the SN-38 treated cells are presented.
Figure 8-9. γ-H2AX image analysis of PBLs treated with A) DMSO control and B) 5 μM SN-38 for 1 hour. The Alexa Fluor™ Image (green) has been superimposed on the DAPI image (blue) using imageJ software.

Due to difficulties with counting individual foci and the fact that measurement of the area staining positive for γ-H2AX per cell is not an established method, this assay was only performed on samples from 2 patients (Figure 8-10). Another disadvantage of this method was that it only assessed adherent cells; as PBLs are usually suspension cells, only a small proportion (that may not have been representative of the whole PBL population for each patient) adhered and were therefore evaluated. Patient 033 had repaired the initial damage (albeit only low levels), as measured by counting γ-H2AX foci, by 4 hours and this patient also developed PD. This was in keeping with ACA findings and thus supported the hypothesis that those patients with PD would be more able to repair the SN-38 induced damage.

Patient 034 still had evidence of DSB detected using γ-H2AX foci present following 10 hours of treatment even though the ACA measured damage had returned to base line.
This suggested that measuring γ-H2AX foci may be a more sensitive method of detecting damage in this setting than using the ACA.

Figure 8-10. Graphs to show the DNA damage induced in PBLs treated with 0 and 5 μM SN-38 ex vivo over a 10 hour time course measured using A) γ-H2ax foci scoring and B) ACA

In order to improve detection of γ-H2AX in PBLs, the alternative method of measuring the total γ-H2AX fluorescence intensity for each cell using flow cytometry was performed (sections 1.8.3.2 and 3.6) initially on PBLs from healthy volunteers (Figure 8-11).
These results confirmed that this method was appropriate to measure an SN-38 induced dose response in PBLs and could therefore be used to assess clinical samples. The dose selected for a time course γ-H2AX assay was 0.1 μM SN-38 on the basis that a) this dose induced measurable but not saturated γ-H2AX levels on healthy volunteers and b) an interim analysis of the ACA data demonstrated that 0.1 μM SN-38 had the strongest correlation with toxicities to treatment (although this was not born out in the final analysis). Seven patients had both ACA and γ-H2AX flow cytometry dose response and time course data available (raw data is shown in Table 8-1 and Table 8-2, corrected data is illustrated in Figure 8-12).
Table 8-1. *Ex vivo* study results: Raw data demonstrating the dose response of PBLs treated with SN-38 for 1 hour *ex vivo* assessed using the flow cytometry measurement of γ-H2AX.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Relative mean fluorescence per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM SN-38</td>
</tr>
<tr>
<td>036 <strong>28</strong></td>
<td>22.67</td>
</tr>
<tr>
<td>037</td>
<td>35.92</td>
</tr>
<tr>
<td>038</td>
<td>30.81</td>
</tr>
<tr>
<td>039</td>
<td>27.68</td>
</tr>
<tr>
<td>040</td>
<td>27.57</td>
</tr>
<tr>
<td>041</td>
<td><strong>27.62</strong></td>
</tr>
<tr>
<td>042 <strong>28</strong></td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 8-2. *Ex vivo* study results: Raw data demonstrating the γ-H2AX levels measured using flow cytometry in PBLs treated with 0 and 0.01 μM SN-38 *ex vivo* over a 24 hour time course.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Relative mean fluorescence per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>0 μM SN-38</td>
</tr>
<tr>
<td>036 <strong>28</strong></td>
<td>22.67</td>
</tr>
<tr>
<td>037</td>
<td>35.92</td>
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<td>038</td>
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<td>039</td>
<td>27.68</td>
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<tr>
<td>040</td>
<td>27.57</td>
</tr>
<tr>
<td>041</td>
<td><strong>27.62</strong></td>
</tr>
<tr>
<td>042 <strong>28</strong></td>
<td>N/A</td>
</tr>
</tbody>
</table>

NB/ For Table 8-1 and Table 8-2: N/A is result/sample not available. Those in **bold** font experienced grade 3/4 toxicities (diarrhoea or neutropenia). Those highlighted had a best response of progressive disease. **28** = UGT1A1*28 homozygote.

When comparing samples, it was important to correct for background fluorescence and non-specific antibody binding therefore all data were corrected by calculating the fold increase in fluorescence from the 0 μM dose for each time point (Figure 8-12).
Figure 8-12. *Ex vivo* study results: Corrected data demonstrating A) dose response and B) time course results of the γ-H2AX levels measured using flow cytometry in PBLs treated with SN-38 *ex vivo*. The fold increase in fluorescence was calculated by dividing the relative mean fluorescence per SN-38 treated cell with the relative mean fluorescence per untreated cell at each time point (unavailable for 1 hour in patient 042).

The γ-H2AX dose response results demonstrated that, as with the ACA, there was an initial increase in DNA damage in all samples followed by a plateau, however response differed in that it saturated at a lower dose than the ACA (1 μM versus 2.5 – 5 μM).

The time course data showed that the maximum damage tended to occur later when measuring γ-H2AX (typically 4 – 10 hours) than when using the ACA (1 hour). As with the AA8 cells, this could potentially be explained by the theory that the majority of the damage detected using the ACA at 1 hour was due to SSBs and the later damage was due to unrepaired DSBs. There was no apparent correlation of the γ-H2AX results with the toxicities to treatment or UGT1A1*28 status (Figure 8-12), however, there was a
trend that those who developed PD had a lower corrected magnitude of damage on both the dose response and time course experiments but these findings were insignificant. Likewise, as the clinical data for the majority of patients having γ-H2AX analysis was censored (due to short follow up times because of their relatively late recruitment on to the clinical study) survival information was still unavailable but these early results once again supported the theory that rapid DSB repair or indeed low levels of DSB induction may predict resistance to treatment.

One might have expected that DNA damage levels detected using the ACA and γ-H2AX assay for each patient would be in proportion to each other i.e. high levels of SSBs would be associated with high levels of DSBs induced by an equal SN-38 dose and vice versa, but this was not shown (Figure 8-13 A). In addition, as DSBs are only formed after the SSBs one would expect that a high level of ACA damage at 1 hour would positively correlate with high levels of γ-H2AX at the later time points however this was also not demonstrated (Figure 8-13 B-D); likewise there was no association of the ACA and γ-H2AX results from identical later time points (Figure 8-13 E-F).
Figure 8-13. Representative scatter plots, investigating the correlations between \textit{ex vivo} SN-38 induced DNA damage in PBLs, measured using the ACA and by the detection of γ-H2AX using flow cytometry. Data are presented as a fold increase in damage from the 0 μM SN-38 control. Figure 8-13 A only shows a correlation between the 2.5 μM dose at 1 hour however there was no significant correlation for any of the doses used. It must be noted that the data shown in Figure 8-13 B-D do not compare equal SN-38 treatment doses; the ACA SN-38 treatment concentration illustrated was 2.5 μM as this gave high DNA damage levels and thus clearly demarcated inter-individual differences and, for reasons detailed in section 6.2.7 and above, the time course doses available for comparison in Figure 8-13 E-F were 5 μM SN-38 for the ACA and in Figure 8-13 B-F 0.1 μM for measurement of γ-H2AX.
No evidence was therefore provided that SSB levels actually predicted the formation of the more toxic DSBs, thus providing an alternative explanation for the lack of strong correlations of the ACA results with the clinical data.

The reproducibility of the γ-H2AX flow cytometry method was assessed by repeating a short dose response and time course experiment on PBLs for the same individual 4 times (Table 8-3). This technique yielded more consistent results than the ACA (section 8.2.1.2)

Table 8-3. A table to demonstrate the reproducibility of measuring the γ-H2AX formation in PBLs following ex vivo treatment with SN-38 by flow cytometry.

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>Dose of SN-38 (μM)</th>
<th>Co-efficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>0.1</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.04</td>
</tr>
<tr>
<td>4 hours</td>
<td>0.1</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.18</td>
</tr>
</tbody>
</table>

8.2.2.4 Apoptosis

The final theory to explain the decline in DNA damage over time to be investigated was that the most damaged cells apoptosed, leaving only the less damaged cells available for analysis at the later time points. Apoptosis in PBLs treated with SN-38 ex vivo was investigated using several methods as detailed below:

8.2.2.4.1 Trypan blue exclusion assay

This assay did not reveal that significant loss of viability was occurring during the 10 hour time course that the ACA was performed in the clinical study however significant cell death had occurred following 24 hours of 5 μM SN-38 exposure (Figure 8-14).
Figure 8-14. A bar graph to show the percentage viability of PBLs following 5 μM SN-38 exposure *ex vivo* assessed by trypan blue exclusion over a 72 hour time course. Results are the mean from 3 independent experiments. Error bars represent the standard deviation. P values were calculated using the independent samples T test.

8.2.2.4.2 Presence of a sub G₁ peak on cell cycle analysis

The proportion of cells in the sub G₁ peak was shown to increase in a dose dependant fashion within 4 hours of SN-38 exposure indicating that apoptosis due to the drug treatment was occurring (p=0.007 calculated using the Chi squared test for trend) (Figure 8-15).
Figure 8-15. Apoptosis induced in PBLs following ex vivo SN-38 treatment, assessed by measurement of the sub G1 peak. Figure 8-15 A is a bar graph to show the percentage of apoptotic PBLs following exposure to 0, 0.1 and 5 μM SN-38 for 1 and 4 hours ex vivo. Data are the mean from 3 independent experiments. Error bars represent the standard deviation. Figure 8-15 B-D illustrate representative histograms obtained using ModFit software following exposure to B) 0, C) 0.1 and D) 5 μM SN-38 ex vivo for 4 hours.
Potential confounders of these data include that: a) dead cells can compromise the accuracy of flow cytometry analysis primarily due to their increased auto-fluorescence (Zamai et al., 1993) and b) interpretation of results is complicated if cell death is also accompanied by G₀-G₁ arrest; thus by the later time points it was not possible to accurately distinguish the cells in G₀-G₁ from the sub G₁ peak. Indeed, even interpreting the data from earlier time points presented in Figure 8-15, the sub G₁ peaks were not clearly resolved and may have been confounded by co-existent G₀-G₁ arrest.

To investigate whether apoptosis, detected using this technique, influenced the ACA results, correlations between the ACA and cell cycle data following 4 hours of SN-38 treatment were sought but no significant associations with either 0.1 or 5 μM doses were demonstrated (Figure 8-16).

![Figure 8-16. Scatter plots to investigate the association of apoptosis as determined by the presence of a sub G₁ peak with DNA damage measured using the ACA in PBLs treated with A) 0.1 and B) 5 μM SN-38 ex vivo for 4 hours. Samples were obtained from a single individual on 4 separate occasions. P value was calculated using the Pearson correlation co-efficient.](image-url)
Although these data supported the presence of apoptosis, the theory that the decline in DNA damage over time as measured using the ACA was due to death of the most damaged cells was not confirmed. The effect of apoptosis on the ACA data was still unclear.

8.2.2.4.3 Annexin

Apoptosis was further investigated using the annexin assay. Significant apoptosis of SN-38 treated cells relative to controls was again demonstrated by 4 hours. At 10 hours of SN-38 exposure cells were shown to have moved from early to late apoptosis and this was even more marked by 24 hours. The observation that the 1 hour viability was higher in SN-38 treated cells was probably a spurious result possibly due to fluorescence of the SN-38 (Figure 8-17).
Figure 8-17. Using the annexin assay to assess apoptosis in PBLs treated with 5 μM SN-38 ex-vivo over a 24 hour time course. P values were calculated using the independent T-test to compare number of viable PBLs in the SN-38 treated samples versus the 0.196% DMSO controls.

As with the presence of the sub G₁ peak, correlations of the annexin data with the ACA data were sought. There a suggestion that an increased percentage of apoptotic cells was associated with lower levels of DNA damage measured using the ACA but this was not significant (p=0.074) for the 5 μM SN-38 dose. Therefore, in contrast to the sub G₁ results, these annexin data did indicate that the decline in DNA damage measured using the ACA over time may indeed be due to apoptosis of the most damaged cells. These results also demonstrated that there was intra-individual variation in the amount of apoptosis occurring per experiment (Figure 8-18).
Figure 8.18 Scatter plots to investigate the association of the presence of apoptosis as determined by the presence of a sub G1 peak with DNA damage measured using the ACA in PBLs treated with A) 0.1 and B) 5 μM SN-38 ex vivo. Samples were obtained from a single individual on 4 separate occasions. P values were calculated using the Pearson correlation co-efficient.

8.2.2.4.4 Association of apoptosis with ACA data

These 3 assays all confirmed that cell death occurred in PBLs as a consequence of ex vivo SN-38 exposure. Both the annexin and sub G1 results demonstrated that apoptosis was occurring within only 4 hours of drug treatment (i.e. the time frame over which the clinical ACA results demonstrated a decrease in DNA damage over time). The effect of apoptosis on DNA damage levels may have therefore have confounded data analysis and thus contributed to the lack of strong associations of the ACA results with the clinical data.

The exact effect of apoptosis on the ACA data may have varied at different time points. It is possible that at earlier time points the DNA degradation associated with cytotoxicity would have led to increased DNA damage, whereas at the later time points the DNA from the most damaged cells may have almost completely degraded prior to
analysis, thus being undetectable and leading to lower than actual damage levels being measured.

There was no correlation of the sub $G_1$ data and the ACA results; however, there was a suggestion, although not statistically significant ($p=0.074$) that apoptosis measured using the annexin assay may correlate with lower levels of DNA damage over time. The reason for the inconsistency in correlations of apoptosis with ACA results may be accounted for by difficulties in interpreting the cell cycle data, due to likely $G_0$-$G_1$ arrest occurring alongside apoptosis and thus the annexin assay may be less prone to error in this situation.

### 8.2.2.5 Cell cycle

In addition to observing that DNA damage induced in PBLs following *ex vivo* SN-38 treatment reduced over time, it was also noteworthy that no new damage appeared to be induced despite ongoing drug exposure. Although deactivation of the SN-38 could account for this (sections 8.2.2.1 and 8.2.2.2), another possible explanation was that unrepaired DSBs may lead to cell cycle arrest thus preventing cells from re-entering $S$ phase and blocking the formation of new SSBs (section 1.6.3.2).

Preliminary studies performed on the CHO DSB repair deficient (irs1SF) and proficient (AA8) cell lines did not provide data to support this theory (section 8.2.2.3). Although the irs1SF cells were shown to arrest in $G_2M$ in the presence of unrepaired DNA damage, results from the AA8 cells demonstrated that ACA measures of DNA damage still declined over time of SN-38 exposure, even though the cells continued to cycle (Figure 8-6 and Figure 8-8).

Cell cycle investigations were also undertaken on PBLs; following 72 hours in culture, cells were treated with either 5 μM SN-38 or control conditions of 0.196% DMSO. The
only significant difference between the controls and the SN-38 treated samples was that at 4 hours, a small yet significantly higher proportion of treated cells were in phase G\textsubscript{2}M (8.45% versus 3.85%, p= 0.011) (Figure 8-19). There was no significant change in the percentage of cells in S phase thus it was unlikely that cell cycle disturbance greatly influenced the ACA results. There were however limitations using this assay (discussed in section 8.2.2.4).

![Figure 8-19](image-url)

Figure 8-19. A graph to demonstrate the cell cycle distribution of viable PBLs (apoptotic cells excluded) following either 0 or 5 μM SN-38 exposure ex vivo over a 10 hour time course. Results are the mean from 3 independent experiments. Error bars represent the standard deviation. * is statistically significant with p calculated using the independent samples T-test.

8.2.3 Assessment of PBLs as surrogates for studying irinotecan and/or SN-38 effect

Another explanation for the lack of strong correlations of the laboratory with the clinical data was that PBLs were not an appropriate normal tissue or tumour surrogate in which to investigate irinotecan effect as evidenced by:
● Ex *vivo* irinotecan treatment of PBLs only induced minimal DNA damage compared to an equipotent dose of SN-38.

● Ex *vivo* SN-38 induced DNA damage did not demonstrate any strong correlations with toxicities to treatment.

● There were no correlations of the polymorphism (UGT1A1*28) known be associated with slow metabolism of SN-38, with DNA damage induced by ex *vivo* SN-38 exposure in PBLs.

● A UGT1A1 inhibitor did not prolong the duration of ex *vivo* SN-38 induced DNA damage in PBLs over a time course.

A series of qualitative analytical experiments were therefore performed to ascertain whether the metabolism of irinotecan and its metabolites by PBLs ex *vivo* was representative of the metabolic pathways that are known to exist in *vivo*; specifically the ability of PBLs to hydrolyse irinotecan and/or glucuronidate SN-38 was assessed. Metabolites were extracted from PBLs following their ex *vivo* exposure to irinotecan or SN-38 and the presence of irinotecan, SN-38 and SN-38G was determined using high performance liquid chromatography fluorescence detection (HPLC-FL) and liquid chromatography mass spectrometry (LC-MS). If these experiments did confirm, as suspected, that metabolism of irinotecan in PBLs did not represent metabolism in the body, then this would substantiate the evidence that this is not a good predictive test of irinotecan toxicity.
8.2.3.1 HPLC-fluorescence (HPLC-FL) detection

8.2.3.1.1 Analysis of the standards

Initially the samples were analysed using a HPLC–FL method before proceeding with analysis using a LC-MS method. An authentic SN-38 standard was used for developing and optimising the method. The SN-38 standard eluted with a retention time of 8.7 min (Figure 8-20 B and C). A small peak corresponding to SN-38 was also observed in the solvent blank which was due to carry over contamination between the HPLC runs (Figure 8-20 A).
Figure 8-20. Typical HPLC-FL chromatograms for the analysis of A) the solvent blank (20mM ammonium acetate pH 3.5/acetonitrile (80:20, v/v)) B) 1 pmol of the SN-38 standard and C) 10 pmol of the SN-38 standard.
8.2.3.1.2 Analysis of metabolites extracted from SN-38 treated PBLs

Following the establishment of the HPLC-FL method using the authentic SN-38 standard, the metabolite extracts from treated PBLs were analysed. Whilst no authentic standard of the SN-38 glucuronide (SN-38G) was available, it was anticipated that if present in the cell extracts the SN-38G peak would elute from the column before the SN-38 peak due to its higher hydrophilicity compared to SN-38 (Sparreboom et al., 1998). A supra-physiological treatment dose of 5μM for SN-38 was used to ensure that any metabolites that may be present were at a high enough level to allow detection by the HPLC-FL system.

SN-38 was detected with a retention time of approximately 8.5 min in the SN-38 treated cells (Figure 8-21 B and C). The identity of this peak was confirmed by co-elution with the authentic SN-38 standard. There was no significant difference in the amount of SN-38 (as determined by the peak intensity) detected in cells that had been treated for 6 h compared to those treated for 24 h. An additional peak was observed at approximately 5.6 min which was not present following the analysis of the authentic SN-38 standard. However, this peak was also present in the DMSO cell control implying that it was not a metabolite related to SN-38. Similar to the situation for the analysis of the SN-38 standard, a small amount of SN-38 carry over was observed in the blank runs using the DMSO control between the analysis of the cell extract samples (Figure 8-21 A).
The HPLC-FL method provided confirmation that SN-38 was present in cell extracts from PBLs following their exposure to the drug but no evidence was obtained for the
presence of the SN-38G metabolite. Further analyses were performed by using a LC-MS method which conferred the advantage of providing structural information of the analyte under investigation when compared to the HPLC-FL method.

8.2.3.2 Liquid chromatography-mass spectrometry (LC-MS)

8.2.3.2.1 SN-38 and irinotecan internal standards

Since a standard for the SN-38G was not available, the mass spectrometer tuning parameters were optimised using the authentic SN-38 and irinotecan standards. Full scan MS analysis of the SN-38 standard showed the presence of an ion at \( m/z \) 393.46 corresponding to the protonated molecule ion ([M+H]+) expected for SN-38. Following MS/MS analysis of the SN-38 standard the major product ion formed from the SN-38 [M+H]+ precursor ion (\( m/z \) 393) was at \( m/z \) 349 corresponding to the loss of \( \text{–CO}_2 \) (Figure 1-3 A).

Similarly for the irinotecan standard, full scan MS analysis showed the presence of an ion at \( m/z \) 587.38 corresponding to the protonated molecule ion ([M+H]+) expected. Following MS/MS analysis, the major product ions formed from the irinotecan [M+H]+ precursor ion (\( m/z \) 587) were at \( m/z \) 124, 195, 167, 110, 543 and 502 (Figure 1-3 B).
Figure 8-22. MS analysis of the A) SN-38 and B) irinotecan standards diluted with 0.1% formic acid/acetonitrile (75:25, v/v) the full scan MS spectrum and the MS/MS product ion spectrum.
The SRM mode transitions used for the detection of SN-38 and irinotecan were \([\text{M+H}]^+ m/z\) 393 to 349 and \([\text{M+H}]^+ m/z\) 587 to 543, respectively. The unavailability of a SN-38G standard meant that the SRM mode transition used for its detection, which was \([\text{M+H}]^+ m/z\) 569 to 393, was obtained from the literature (Santos et al.).

The typical LC-MS/MS SRM ions chromatograms for the detection of SN-38 and irinotecan standards are shown in Figure 8-23 A and B. Irinotecan and SN-38 eluted with retention times of 7.5 and 15.7 minutes respectively. It should be noted that a relatively small peak was observed at 7.5 minutes for the irinotecan standard in the SN-38 SRM channel which may be attributable to in source fragmentation of the irinotecan, and in addition a small amount of SN-38 carry over was detected at 15.64 minutes (Figure 8-23 B).
Figure 8-23. Typical LC-MS/MS SRM ions chromatograms for A) the SN-38 standard (10 pmol) and B) the irinotecan standard (10 pmol).
8.2.3.2.2 Analysis of the glucuronidation capacity of PBLs following treatment with SN-38 ex vivo

Two different SN-38 dosing treatments were used to investigate the ability of PBLs to produce glucuronide metabolites. A low dose (0.05 μM) reflecting the physiological dose and a higher dose (5 μM) were used to treat the PBLs. The typical LC-MS/MS SRM ions chromatograms for the detection of SN-38 in the low and high dose treatments for 4 hours are shown in Figure 8-24 B and C. SN-38 was clearly detectable in the 5 μM treated samples, however for the 0.05 μM treated cells the size of the SN-38 peak detected was greatly reduced (Figure 8-24 B shown by the arrow) and is at the limit of detection of the LC-MS/MS method. A peak eluting at approximately 3.7 min was observed in the SN-38 G channel for both dose treatments, however a peak of similar intensity and retention time was also observed for the DMSO control treated cells (Figure 8-24 A) indicating that it was not a SN-38 G metabolite. Further analysis of the 3.7 min peak by LC-MS/MS showed that no product ions related to SN-38 G were detectable in the product ion spectrum and alterations of the initial gradient conditions did not affect the retention time of the peak indicating that it was a polar contaminant present in the DMSO. Similar LC-MS/MS SRM ions chromatograms were obtained for PBLs from a second donor treated with 5 μM SN-38 for 6 and 24 h (Figure 8-25 A and B). A peak corresponding to SN-38 was clearly detectable for both time points but no peaks were detected for the SN-38G metabolite.
Figure 8-24. Typical LC-MS/MS SRM ions chromatograms for PBL cell extracts from donor 1 treated with A) DMSO control B) 0.05 μM SN-38 for 4 hours and C) 5 μM SN-38 for 4 hours.
Figure 8-25. Typical LC-MS/MS SRM ions chromatograms for PBLs cell extracts from donor 2 treated with 5 μM SN-38 for A) 6 hours and B) 24 hours.
8.2.3.2.3 LC-MS/MS SRM analysis of PBLs treated with irinotecan *ex vivo*

The typical LC-MS/MS SRM ions chromatograms obtained for the analysis of PBLs treated with irinotecan *ex vivo* are shown in Figure 8-26. A peak corresponding to SN-38 was detected at 15.55 min following the *ex vivo* exposure of PBLs to 50 μM irinotecan for 4 hours indicating that PBLs have the capacity to metabolise irinotecan *ex vivo*. The peak present in the SN-38 channel at 7.82 min was once again due to source fragmentation of the irinotecan (Figure 8-26 C). To confirm that the SN-38 peak detected was not due to carryover, a series of solvent blank injections were performed prior to injection of the test sample for which no peaks were observed (Figure 8-26 A). It should be noted that a relatively small peak was observed at 7.7 min corresponding to irinotecan in PBLs exposed to DMSO *ex vivo* which may be attributable to contamination of the DMSO (Figure 8-26 B).
Figure 8-26. Typical LC-MS/MS SRM ions chromatograms for A) solvent blank (20mM ammonium acetate pH 3.5/acetonitrile (80:20, v/v)) B) PBL cells treated ex vivo with DMSO (control) and C) PBL cells treated ex vivo with 50 μM irinotecan for 4 hours.
Overall, this metabolism data provides evidence that PBLs are not a good surrogate for predicting the effects of irinotecan. The LC-MS results demonstrated that the PBLs converted only a very small proportion of irinotecan to SN-38. This observation was consistent with the low levels of CES activity that were previously reported in the literature (Chazal et al., 1996, Guemei et al., 2001) and provided confirmation that the reduced ACA dose response of PBLs treated directly with irinotecan ex vivo was due to the inefficient production of SN-38.

There was no evidence for the occurrence of SN-38 glucuronidation in PBLs ex vivo which is in contrast to the glucuronidation of benzo[a]pyrene diols and diones that has been previously reported (Hu and Wells, 2004) and is most likely explained by the requirement of different UGT enzymes. The toxicity of irinotecan treatment is due to the slow metabolism, therefore the over-accumulation of SN-38 and the absence of
glucuronide metabolites may in part explain why DNA damage measured using the ACA in the clinical trial did not correlate with irinotecan toxicities.

8.3 Conclusion

These mechanistic experiments confirmed that there were some shortcomings when using the ACA to detect *ex vivo* SN-38 induced DNA damage. It is therefore not possible to reject the hypothesis that DNA damage induced in PBLs is a predictive biomarker of irinotecan effect because if these shortcomings could be overcome then stronger correlations with some of the clinical data may potentially be sought.

There were high levels of both intra- and inter-patient variation in the ACA results which were most likely due to the influence of several processes including cell division, apoptosis and DNA repair on the levels of DNA damage detected. The complex interplay of these factors, each occurring at different rates and having conflicting effects on the DNA damage levels is likely to have contributed to the lack of significant associations with the clinical data.

In addition to the ACA not being the optimal method used to detect DNA damage, experiments have also provided evidence that PBLs may not be an appropriate surrogate in which to investigate this drug’s effect. LC-MS data have shown that the *ex vivo* metabolism of irinotecan and SN-38 in PBLs does not mimic what is known to occur *in vivo*. As toxicities to treatment are due to the over-accumulation of SN-38, and PBLs do not glucuronidate this metabolite *ex vivo*, one can therefore conclude that SN-38 induced DNA damage in PBLs is not a predictive biomarker of toxicities to treatment which is in keeping with the lack of correlation in the clinical study results.
There were however data demonstrating that DNA damage may be a biomarker of irinotecan response as illustrated by the significant association of high levels of DNA damage at 4 and 10 hours with improved PFS in the clinical trial participants. DSB repair studies have provided evidence that unrepaired DSBs contribute towards the persisting damage measured using the ACA at these later time points and thus efficient DSB repair may predict poor PFS. There was no evidence that ACA data predicted the formation of the more toxic DSBs as shown by the lack of correlation between DNA damage measured using the ACA with that measured using the detection of γ-H2AX. In view of this observation and the fact that the measurement of γ-H2AX has been shown to yield more reproducible results when treating PBLs ex vivo than the ACA, additional work, using this test to further define the association of DSB induction and repair with survival and response is warranted.
9 General discussion

A summary of the overall objectives of this thesis and results demonstrating how these were achieved are presented below for clarification. Then follows a discussion of the advantages and limitations of the research methods used and finally, to conclude, a proposal for how the positive results from this study could be pursued in the future.

9.1 Summary

Irinotecan is one of the three most important cytotoxic drugs prescribed for the treatment of metastatic CRC, however its use is limited by the presence of unpredictable toxicities and response, which are known to occur largely due to inter-individual variations in the interplay of the many proteins involved in its metabolism. Irinotecan is currently prescribed using a patient’s body surface area, at doses derived from clinical trials based on outcomes across populations. This approach does not account for inter-individual differences in pharmacokinetics. Prior to commencing this research, the need for a predictive test of irinotecan response and/or toxicities was well recognised as illustrated by the plethora of articles in the medical literature detailing attempts to develop such a technique, none of which had proved adequate to alter routine clinical practice (chapter 1). One major weakness of these predictive tests previously investigated was they failed to account for all of the enzymes, transporters and environmental factors that are (known and unknown) to be involved in this drug’s complex metabolism.

Presented in this thesis are the design, conduct and mechanistic analysis of the first prospective clinical study performed to assess whether DNA damage induced in PBLs following irinotecan or SN-38 exposure is a predictive biomarker of irinotecan effect.
DNA damage is a key, biologically significant event that takes into account many genetic and epigenetic influences on a cell. This research hypothesis was made on the basis that irinotecan exerts its cytotoxicity by stabilising the complex formed between topo I and DNA, thus inducing SSBs which are subsequently converted to more toxic, irreversible DSBs. Therefore, as DNA damage is an end measure of irinotecan effect in the cell, this method had a theoretical advantage over other techniques previously investigated, of being able to account for all factors affecting irinotecan metabolism and its binding to topo-I.

The demographics of the 42 clinical trial participants are presented in chapter 4 and were comparable to the metastatic CRC populations investigated in large multi-centre phase 3 studies. Data demonstrated that 40% of the patients recruited would have benefited from a predictive test of irinotecan effect. The most widely described predictive biomarker of irinotecan effect investigated to date, namely homozygosity for UGT1A1*28 was not associated with toxicities to treatment, however, there were insignificant trends that presence of this polymorphism (and thus being slow to metabolise SN-38 to inactive SN-38G) correlated with improved response and survival. The utility of UGT1A1*28 genotyping reported in the literature to date has been inconsistent (section 1.7.2.3.3) and these study data further supported the view that a superior method to predict irinotecan effect is required.

Early clinical study data are presented in chapter 5 and showed that irinotecan induced DNA damage could not be detected in PBLs following drug exposure in vivo. This negative result was confirmed to be due to the fact that PBLs usually reside in phase \( G_0G_1 \) of the cell cycle and for irinotecan / SN-38 DNA induced damage to occur, cells are required to cycle. This observation was subsequently used to develop the ex vivo
clinical trial protocol using mitogenic stimulation of PBLs prior to drug treatment to induce DNA damage that could then be measured using the ACA or by detection of γ-H2AX (chapter 6). Treatment with SN-38 induced higher levels of damage than an equipotent dose of the prodrug irinotecan and this was subsequently demonstrated to be due to the fact that the hydrolysis of irinotecan to SN-38 occurs only inefficiently in PBLs (chapter 7).

The ex vivo study findings showed that DNA damage induced in PBLs did not predict toxicities to irinotecan chemotherapy (chapter 7). This lack of association was purported to be due, at least in part, to the inability of PBLs to catalyse the glucuronidation of SN-38 to inactive SN-38G (chapter 8). There were however data to support the theory that DNA damage may be a biomarker of response and survival to irinotecan treatment (Chapter 7). In particular, those individuals who had low levels of DNA damage at later time-points, believed to be due to efficient repair of the DNA damage had significantly poorer progression free survival. This finding needs to be interpreted with caution due to the relatively small patient numbers studied and the need to apply a correction factor however it does warrant further investigation (section 9.3).

9.2 Research methods used

Although statistical correlations of the ACA results with PFS have been obtained, data analysis in this study has been complex due to the presence of several shortcomings and confounding factors with the methods used. Whilst these methods had some clear advantages, there were drawbacks to performing the ACA, measuring γ-H2AX, using
PBLs as surrogates and interpreting clinical data that were identified and are discussed below.

9.2.1 ACA

The ACA was assay of choice to measure DNA damage as it had several potential advantages including: a) being sensitive to detect low levels of DNA damage at pharmacologically relevant doses, b) being relatively cheap, c) requiring only a small number of cells therefore being appropriate to assess clinical samples, d) having the ability to detect several types of DNA damage and e) yielding results within only a few days.

Whilst the ACA did successfully detect irinotecan / SN-38 induced DNA damage, some shortcomings associated with this method did cause difficulties during the course of this research. One major limitation of the ACA is the existence of variability in results between experiments and centres and as yet, there is no internal standard to correct for this. Results from an inter-laboratory validation trial had previously demonstrated that there was a large variation in the absolute level of DNA damage measured but the laboratories could detect concentration-dependent trends in coded samples (Moller et al., 2010). In this study, experiments to investigate the reproducibility of the ex vivo SN-38 method in PBLs demonstrated that there was intra-individual variation in the results obtained. This variation would therefore have decreased the sensitivity of the assay to detect inter-individual differences and thus may have masked any associations with the clinical data. It is noteworthy that the correlation of the ACA results with the PFS obtained in this study required a correction factor to be applied in order to reach significance. A uniformly accepted internal standard could thus greatly improve the accuracy of this method. Work on developing such a standard consisting of 'reference'
cells which have had their DNA substituted with BrdU is ongoing (Zainol et al., 2009). It may however be that, with the variability as it stands, this method is only of sufficient sensitivity to demonstrate differences between groups of patients but is not robust enough to predict outcome for a given individual.

An additional drawback to this method was the occurrence of apoptosis in PBLs that was confirmed to occur during culture and treatment. There is controversy within the research community about the appearance of apoptotic cells when performing comet analysis. Some believe that the presence of highly damaged “hedgehog” cells with small heads and diffuse tails represent apoptotic cells and thus exclude hedgehogs from the analysis on the assumption that the high levels of DNA damage are due to DNA degradation occurring due to cell death and thus represent a cytotoxic rather than a genotoxic effect (Burlinson et al., 2007). Others dispute this and have demonstrated that hedgehogs may repair and thus by definition cannot be apoptotic (Collins et al., 2008). For consistency, hedgehogs were excluded in this study (Figure 9-1).

![Figure 9-1. An example of a “hedgehog” comet viewed using fluorescence microscopy](image-url)
DNA repair was also demonstrated to be occurring during the time course over which the ACA was performed. In some situations repair may affect the DNA damage levels detected and confuse data interpretation. For example excision repair may increase DNA migration due to incision-related DNA strand breaks. In addition, short lived primary DNA lesions e.g. SSBs which may undergo rapid DNA repair could be missed when using inadequate sampling times (Brendler-Schwaab et al., 2005).

A final disadvantage to consider when contemplating using the ACA in routine clinical practice is that it is labour intensive and thus may not be a cost effective test in a hospital laboratory. In order to improve the throughput of genotoxicity screening, fully automated scoring systems have been developed (Frieauff et al., 2001), although results with these systems to date have been poor and thus have not yet achieved widespread use.

9.2.2 γ-H2AX assay

The measurement of γ-H2AX is regarded as the most sensitive way of detecting DSBs. This method therefore had the theoretical advantage over the ACA of being more specific to detect the most cytotoxic and thus arguably most relevant DNA damaging consequence of irinotecan effect. In addition, compared to the ACA results, the γ-H2AX data were shown to be less subject to variation (section 8.2.2.3.2). When using flow cytometry analysis, this assay was less labour intensive than the ACA and thus would be more appropriate to perform on a large scale in a clinical setting.

However, once again there were disadvantages when using this method. As with the ACA assay, results obtained may have been confounded by the presence of other ongoing processes during drug treatment including, mitosis, apoptosis and repair. Although regarded as a sensitive technique, γ-H2AX detection is not always specific as
foci may form in response to other DNA lesions in addition to DSBs; for example ultraviolet C radiation induces foci formation through ATR kinase activity and γ-H2AX may also form during apoptosis and mitosis in some cell types (reviewed in (Bonner et al., 2008)).

Another limitation is that γ-H2AX foci are only a surrogate for DSB formation. It is therefore difficult to absolutely conclude whether the removal of foci accurately correlate with DNA repair. There are data demonstrating that γ-H2AX foci disappear alongside DSBs detected by the neutral comet assay thus supporting its use to measure repair (Mirzayans et al., 2006). However, conflicting data show the level of foci remain elevated after even after DSB repair, implying that steps that follow DNA rejoining are necessary for foci removal (Kinner et al., 2008).

9.2.3 Using peripheral blood lymphocytes as a normal tissue surrogate

The main advantages and rationale of using PBLs as a “surrogate normal tissue” were that they were readily available and they contained genomic DNA thus in theory their gene expression profiles should have been representative of other normal host tissues. It was anticipated that the main disadvantage would be that the biological effects observed in these surrogate cells may not representative of those occurring in other somatic cells or tumour cells. Indeed, results did confirm that PBLs were not an optimal surrogate in which to investigate irinotecan effect as demonstrated by the fact that they hydrolised irinotecan only weakly and did not catalyse SN-38 glucuronidation at all in comparison to the predominantly hepatic metabolism that is known to occur in vivo. This therefore may have explained why DNA damage in PBLs did not prove to be a biomarker of irinotecan toxicities. An additional theory to account for the lack of association with toxicities is that the SN-38 can be regenerated
from SN-38G by gut bacteria and thus may accumulate causing diarrhoea (Rasmussen et al., 2011). This process would clearly not be accounted for when investigating PBLs. This choice of cell type may also have contributed to the intra-individual variation in ACA results. Previous researchers have demonstrated that only a fraction of PBLs developed comet tails with different intensities following exposure to cytotoxic chemotherapeutic drugs. This inconsistency was believed to be due to the fact that DNA damaging agents would not act equally in a heterogeneous mixture of circulating PBLs comprising of various subpopulations that differed in age, cell cycle status and function (Sanchez-Suarez et al., 2008). Efforts were made to minimise this inconsistency by scoring 50 cells per gel (2 gels per slide) and ensuring each condition was processed in triplicate for each individual. In addition, the manipulation required in this method to ensure that PBLs were cycling using PHA stimulation was a further stage in which variability may have been induced and thus may have masked possible correlations with the clinical data. Alternatively, this manipulation may have been beneficial as it may have ensured that PBLs mirrored actively dividing cells that are prone to toxicities. Also, as freshly isolated lymphocytes can be slow to repair (Collins et al., 2008), it enabled time for them to repair damage obtained during isolation and resume repair capacities more representative of normal cells prior to testing the ex vivo SN-38 effect.

Another potential shortcoming was that normal somatic cells such as PBLs do not have the same sensitivity to chemotherapeutic agents as malignant cells, which develop different chemo-resistance mechanisms. It was therefore interesting that the main significant finding from this study was that DNA damage as measured using the ACA was predictive of progression free survival. This finding was only significant at later
time points and was thought to be most likely due to inter-individual variations in DSB repair. From the results available it is therefore possible that the DSB repair processes detected in PBLs did correlate with the repair within the tumours but this requires further investigation.

A final limitation is that PBLs do not necessarily account for other non-genetic factors (e.g. concomitant medications) that may influence the chance of response or development of toxicities to irinotecan treatment.

9.2.4 Confounding factors in clinical data analysis

Data analysis was facilitated by the fact that, when patients were classed according to the response or toxicities to treatment, these groups were demographically comparable with the notable exception that those experiencing toxicities had a significantly higher average performance status.

The major difficulty with the clinical data analysis was that patients received irinotecan in combination with other drugs, most frequently 5-FU. As toxicities to 5-FU include diarrhoea, and neutropaenia it was not possible to confirm for certain which of the drugs were responsible for the toxicities documented. In addition, the dose of irinotecan administered varied between treatment regimens and patient numbers were not sufficient to allow analysis of each individual regimen used.

9.3 Indications for future work

This thesis describes the first prospective clinical study conducted to assess whether DNA damage is a biomarker of irinotecan effect. Results have confirmed that DNA damage in PBLs does not predict toxicities to treatment thus further pursuing some aspects of this research is unlikely to yield beneficial information. However, there were
also some potential associations with response and survival demonstrated that do warrant further investigation.

Results confirmed that irinotecan or SN-38 induced DNA damage in PBLs is not predictive of toxicities to treatment. This lack of correlation is in part explained by the failure of PBLs ex vivo, to mimic the in vivo metabolism of this drug. It is thus possible that DNA damage induced in an alternative normal tissue surrogate, with metabolism more closely resembling that occurring in vivo, may indeed be a biomarker of toxicities. As hydrolysis of irinotecan and glucuronidation of SN-38 occur primarily in the liver, hepatic tissue would thus be the most likely to yield positive results. This is therefore not a viable avenue to pursue as the risks and discomfort of performing a liver biopsy would outweigh any potential benefits and thus not be justified in this setting. An optimal surrogate normal tissue needs to be easily and safely available with minimum distress to the patients; such tissues include buccal cells, hair follicles, spermatozoa and skin (Rockett et al., 2004). However, the problems demonstrated using PBLs in this study, would also be likely to exist using these other normal tissues. One major difficulty would be that these alternatives are unlikely to yield cells that will undergo cell division in the laboratory. In addition, just as the ex vivo metabolism of irinotecan and SN-38 in PBLs did not reflect the processes occurring in vivo, it is doubtful that these other accessible tissues would express the predominantly hepatic CES and UGT enzymes relevant to irinotecan metabolism either.

Although this method was not successful at predicting the effect of irinotecan on normal tissues, there was evidence that ex vivo SN-38 induced DNA damage in PBLs, measured using the ACA, could predict tumour response to irinotecan treatment. This was illustrated by the trend that the dose response curve was more likely to saturate
at low SN-38 doses in patients who responded to treatment, and those with progressive disease would demonstrate a plateau at higher doses. Low sensitivity limited the potential clinical utility of this finding, therefore to assess whether this can be improved, a more detailed assessment, using additional doses ranging between 2.5 – 5 μM SN-38 in further patients is warranted (section 7.2.1.1).

A study of irinotecan/SN-38 induced DNA damage on CRC cells obtained from patient samples would be academically interesting but the clinical utility would be limited, because irinotecan treatment is usually commenced when patients have known their diagnosis for some time, thus would not have a clinical indication to undergo a biopsy.

However, if CRC cultures could be obtained using a minimally invasive procedure, for example from circulating tumour cells (CTCs) obtained from blood samples using antibody-based assays or molecular methods (a so called “blood biopsy”) (Barok and Szollosi, 2011), then the assessment of whether ACA measures of irinotecan/SN-38 induced DNA damage correlate with response to treatment would be justified. CTCs are the subject of much current research and their detection and quantification has already demonstrated a prognostic significance in metastatic CRC (Cohen et al., 2009). A potential problem is that CTCs form a heterogeneous population of cells with biological characteristics often different from those of their respective primary tumour progenitors. However, pilot studies have shown that their phenotyping could be used to predict response to targeted therapies (Mavroudis, 2010). If CTCs were to be investigated as a predictor of irinotecan response, an anticipated difficulty in developing the assay would be to establish conditions whereby the CTCs could be cultured following their isolation.
DNA damage was significantly associated with progression free survival although these findings were only significant once correction factors derived from irradiated controls were used. This illustrates that the main limitation when using the ACA is its intrinsic variability (Moller et al., 2010) and thus the introduction of a widely accepted internal standard may help to improve this (Zainol et al., 2009). If a reliable internal standard becomes available, a repeat assessment of the ACA time course experiment in a further clinical study could be conducted. The aim would be to accurately identify a specific level of DNA damage, below which the PFS would be so poor, that individuals unlikely to gain sufficient benefit from treatment to justify the toxicity risk, could be identified. A clinical study comparing a) personalised allocation of irinotecan excluding those with predicted poor PFS with b) standard allocation treating all deemed medically suitable, would only be deemed feasible if a highly predictive specific level of DNA damage was identified.

A further assessment of the γ-H2AX assay on greater patient numbers is also justified as this assay was demonstrated to have increased reproducibility compared to the ACA in this setting, but patient numbers were not sufficient to confirm or refute its potential clinical utility.

The association of increased corrected ACA damage at 4 and 10 hours of drug exposure with improved PFS also highlighted a likely role of DNA repair in acquiring resistance to this drug. Further genetic studies could help to identify repair genes that may lead to resistance to irinotecan treatment although, the largest biomarker study conducted to date in metastatic CRC did not show any predictive value of the two DNA repair genes studied (XRCC1 and MLH1) with irinotecan outcome (Braun et al., 2008, Braun et al., 2009).
Individualisation of irinotecan treatment using robust, evidence-based prediction of efficacy and toxicity remains a desirable goal but further research is needed. The majority of recent research into irinotecan effect has focussed primarily on genotyping, but this study has explored a novel alternative. Measuring DNA damage has not been shown to be predictive of toxicities, thus ongoing exploration of genotyping is probably more likely to successfully achieve a method of personalising the irinotecan dose prescribed. However, with refinement there is the potential that DNA damage may improve the prediction of prognosis, and aid the identification of those who may not benefit from treatment and thus could be spared unnecessary toxicities from this drug.
10 Appendix: Communications at scientific meetings


Irinotecan is a topoisomerase I inhibitor used to treat metastatic colorectal cancer. Its use is limited by the heterogeneity in both its toxicities and clinical response which are currently unpredictable for the most commonly prescribed doses. We aim to establish whether DNA damage is a predictive biomarker of this drug’s effect.

A prospective clinical study is being conducted whereby peripheral blood lymphocytes (PBLs) are being isolated from patients before they receive irinotecan-based chemotherapy. A method to culture these PBLs with phytohaemagglutinin stimulation and then treat them ex vivo with SN-38 (the active metabolite of irinotecan) to induce DNA damage has been developed. Single strand DNA breaks are being measured using the alkaline comet assay (ACA) and double strand breaks are being assessed by intensity based analysis of γH2AX. Correlations between the DNA damage levels and the clinical toxicity and response data are being investigated.

Results demonstrate that inter-individual differences in induced DNA damage levels exist. An interim analysis performed following recruitment of 30 participants showed that the mean percentage tail DNA following treatment of the PBLs with 0.1 μM SN-38 for 1 hour was insignificantly increased in those who required a dose reduction or cessation of treatment due to toxicities (n=11) compared to those who tolerated treatment well (n=19) (10.3% Vs 8.4%, p = 0.21). Response data demonstrated that the mean percentage tail DNA was significantly higher in those who progressed on treatment (n=3) compared to those who clinically benefited (n=27) (12.5% Vs 8.7%, p = 0.05). γ-H2AX levels have been measured in 4 patients but no significant correlation with the ACA or clinical data has been established.

The heterogeneity in results of these assays and the early correlations with clinical data support the hypothesis that DNA damage may be a predictive biomarker of irinotecan effect.


Background: Irinotecan treatment is limited by the heterogeneity in its toxicities. Although UGT1A1 polymorphisms have been shown to correlate with toxicities this association is only clinically relevant at high doses. No predictive test has yet been developed for the most commonly prescribed regimes. We aim to establish...
whether genotoxic measures of irinotecan effect correlate with tolerance to this agent.

**Method:** A prospective clinical study is being conducted whereby peripheral blood lymphocytes (PBLs) are being obtained from patients before receiving irinotecan chemotherapy. We have developed a novel method to culture the PBLs with phytohaemagglutinin stimulation and then to treat them ex vivo with SN-38 (the active metabolite of irinotecan). This induces DNA damage that can then be detected using the alkaline comet assay. Correlations are being investigated between the DNA damage levels induced using this laboratory assay and the clinical toxicity data from these patients.

**Results:** To date 22 patients have been recruited and 4 of these have required dose reductions of their irinotecan chemotherapy due to toxicities. Dose response experiments have demonstrated that inter-individual differences exist in both the gradient and magnitude of response. Time course experiments have also demonstrated that these ex vivo damage levels reduce at different rates in different individuals. Further mechanistic studies are underway to explain the reasons for these variations but they are most likely due to differences in either inactivation of the SN-38 or repair of the DNA damage. Preliminary toxicity data show a trend towards a longer duration of DNA damage and steeper dose response curves correlating with toxicities.

**Conclusion:** The heterogeneity in results of this ex vivo assay that we have developed and the early correlations with toxicity data supports its further evaluation to deliver a predictive test of irinotecan toxicity. Patient recruitment is ongoing.


**Background:** Up to one third of patients receiving irinotecan treatment experience grade 3 or 4 toxicities. Although UGT1A1 polymorphisms have been shown to correlate with high dose irinotecan toxicity, there is not yet a predictive test in use for the most commonly prescribed doses.

**Aims:** Establish whether genotoxic measures of irinotecan effect correlate with patient tolerance and / or response to this agent. Develop an assay to detect DNA damage following irinotecan exposure in vitro.

**Method:** The alkaline comet assay (ACA) is being used to detect irinotecan induced single strand DNA breaks in peripheral blood lymphocytes (PBLs). DNA damage induced in vivo is being investigated in samples taken from patients prior to, 1 hour and 24 hours following chemotherapy. PBLs from healthy volunteers were studied to optimise assay conditions to induce DNA damage with in vitro irinotecan exposure.

**Results:** Irinotecan induced DNA damage has been detected in vivo in 4/9 of the patients studied so far (maximum 2.88% increase in median percentage tail DNA).
In vitro treatment of PBLs has shown that maximum DNA damage is detected after 1 hour of treatment. Exposure to the active metabolite SN-38 elicits a greater effect than irinotecan. The response is increased if PBLs are cultured using phytohaemagglutinin stimulation (maximum median percentage tail DNA increased by 32%).

**Conclusion:** Only minimal levels of ininotecan induced DNA damage are detectable using the ACA when the drug exposure has occurred in vivo. This is probably because irinotecan exerts its effect in S phase and lymphocytes usually reside in phase G0 of the cell cycle. Higher levels of damage can be induced ex vivo by ensuring lymphocytes are cycling prior to treatment. Sample acquisition is ongoing and correlations of DNA damage levels with clinical data are awaited to assess if this assay is a useful predictive test.

*Joanna Wood, Karen Bowman, Anne L. Thomas, George D.D Jones, Establishing a genotoxic predictive test of irinotecan toxicity in colorectal cancer (Meeting abstract). Medical Research Society, Academy of Medical Sciences and Royal College of Physicians meeting for Clinician Scientists in training, 25th February 2010, London.*

**Background:** Irinotecan is a valuable drug in the treatment of metastatic colorectal cancer but its use is limited by significant heterogeneity in its toxicity. Irinotecan is delivered as a prodrug that is converted to its active moiety SN-38 which induces single-strand DNA breaks (SSBs) by stabilising the complex formed by topoisomerase I and DNA. Although UGT1A1 polymorphisms can correlate with toxicity there is as yet no predictive test in routine use.

**Aim:** To establish whether laboratory genotoxic measures of irinotecan effect correlate with patient tolerance to this agent and so ultimately delivering a clinical predictive test of toxicity.

**Method:** Blood samples are being obtained from patients undergoing irinotecan chemotherapy pre, 1 hour and 24 hours post infusion. Levels of endogenous and irinotecan induced SSBs in peripheral blood lymphocytes (PBLs) are being determined using the alkaline comet assay (ACA). PBLs from healthy volunteers have been studied for the optimisation of the assay to induce and then detect these irinotecan induced SSBs ex-vivo.

**Results:** Of the 6 patients recruited so far, in 3 patients who tolerated treatment well the ACA showed no significant difference between the pre and post samples. In the other 3 patients who experienced grade 3 or 4 toxicities the mean percentage tail DNA 1 hour after treatment increased by between 2% and 6% (p < 0.02). Ex-vivo drug challenges on PBLs from healthy volunteers have shown that maximum DNA damage is detected after 1 hour of treatment and levels are back to baseline by 8 hours. Dose response experiments demonstrate that treatment with the active metabolite SN-38 elicits a greater effect than irinotecan. Response is increased if PBLs are cultured using phytohaemagglutinin stimulation for 72 hours prior to drug exposure (maximum mean percentage tail DNA increased by 32%).


**Conclusion:** Preliminary data suggest that the endogenous DNA damage secondary to irinotecan treatment detected by the ACA correlates with patients’ toxicities. It is possible to induce DNA damage to PBLs using irinotecan or SN-38 *ex-vivo* thus demonstrating potential in developing a predictive test of irinotecan toxicity.
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