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The role of acid ceramidase in the radiotherapy response of an in vitro model of rectal cancer

Submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine

by

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Dedication

Mum, I will never forget everything you did for me. You will always be missed.

To my family, you are the most important people in my life. Thank you for all your unconditional love and continued support to help me get to this point.

I will be forever grateful.

x R M A B H H x

Declaration

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The material contained within this thesis has not been, nor is currently being presented wholly, or in part, for any other degree or qualification.

Narendranath Govindarajah

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“If I have seen further it is by standing on the shoulders of Giants"

Sir Isaac Newton.

Abstract

The role of acid ceramidase in the radiotherapy response of an in vitro model of rectal cancer

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Background: Chemo radiotherapy (CRT) is often employed to treat locally advanced rectal cancer with highly variable response, emphasizing the necessity for predictive response biomarkers. Our initial proteomic and immune-histochemical work demonstrated that acid ceramidase (AC) expression correlated with poorer CRT responses in rectal cancer. We described that higher AC expression correlates with radio resistance in colorectal cancer cells and improved radio sensitivity through siRNA inhibition of AC. The mechanisms behind AC expression, radio resistance and apoptosis remain unknown in colorectal cancer. AC is known to affect apoptosis and the enzyme poly (ADP-ribose) polymerase-1 (PARP-1) is a DNA repair enzyme that is also cleaved into specific fragments during apoptosis.

Aims: To elucidate a potential mechanism linking AC expression with radio resistance in colorectal cancer cells.

Methods: Differential AC protein expression of four colorectal cell lines was confirmed by Western blotting. Radio sensitivity of these cell lines was examined using standard clonogenic assays by counting individual colony survival post-exposure to increasing doses of ionizing radiation. siRNA knockdown of AC was performed with further clonogenic assays to establish the impact of AC inhibition on radio sensitivity. HT29 and HCT cells were then treated with non-targeting control siRNA and AC siRNA, irradiated at increased doses of radiation then harvested at specific time points (2,6,24h). Western blotting was then performed to detect and measure for specific PARP-1 cleavage fragments as specific apoptotic markers.

Results: Clonogenic assays confirmed that cell lines with greater cellular AC protein expression (LIM1215/MDST8) demonstrated higher colony survival compared to those with lower AC expression (HT29/HCT 116) post irradiation. siRNA AC knockdown improved radio sensitivity by reducing colony formation efficiency (CFE) in three cell lines: HT29(0.52 CFE control vs 0.13 CFE knockdown at 1Gyp=0.00004); HCT116(0.24 CFEcontrolvs0.09 CFE knockdown at 1Gyp=0.026); LIM1215 (0.88 CFE control vs 0.43 CFE knockdown at 0.25Gyp=0.001).Western blotting confirmed that HT29,HCT116 and LIM1215cells treated with AC siRNA displayed significantly higher levels of the 24kD PARP-1 cleavage fragments compared to control therefore indicating increased apoptosis.

Conclusion: Higher AC expression correlates with radio resistance in several colorectal cell lines and radio sensitivity was successfully improved through biological (siRNA) inhibition of AC. Initial mechanistic work has confirmed that siRNA inhibition of AC causes increased apoptosis in multiple cell lines following ionizing radiation; this could suggest a role of AC mediating radio resistance through preventing irradiation-induced apoptosis. This work further solidifies AC as a target for improving radiotherapy treatment of locally advanced rectal cancer.

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Abbreviations

5-FU 5-Fluorouracil

AC acid ceramidase

AJCC American Joint Committee on Cancer

APC adenomatous polyposis coli

APHRODITE A Phase II trial of Higher RadiOtherapy Dose In The Eradication of early rectal cancer

APR abdominal perineal resection

ASAH1 N-Acylsphingosine Amidohydrolase 1

Bax BCL2-associated X protein

Bcl-2 B-cell CLL/lymphoma 2

Bcl-2 B-cell lymphoma

BRAF v-raf murine sarcoma viral oncogene homolog B1

CA19-9 carbohydrate antigen 19-9 cCR complete

clinical response CD95 apoptosis antigen

CDKs cyclin dependant kinases

CDX2 homeobox protein CDX-2 CEA

carcinoembryonic antigen cfDNA

circulating free DNA

CIMP CpG island methylator phenotype

CIN chromosomal instability

CK cytokeratin

CNS central nervous system

COPERNICUS Chemotherapy then Radiation then Immediate Curative Surgery for operable rectal cancer

COX-2 cyclooxygenase-2

CPG 5'—C—phosphate—G—3'

CRC colorectal cancer

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

CRM circumferential resection margin

CRT chemo-radiotherapy

CSC cancer stem cell

CT computerised tomography

Cyt c cytochrome c

DCC netrin receptor DCC

DMEM Dulbecco’s modified Eagle’s medium

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DSB double-strand break

DTT DL-Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

EGFR Epidermal growth factor receptor

EORTC European Organisation for Research and Treatment of Cancer

EPCAM epithelial cell adhesion molecule

FAP familial adenomatous polyposis

FasL Fas ligand

FFCD Fédération Francophone de Cancérologie Digestive

FIT faecal immunochemical test

FOB faecal occult blood testing

FU fluorouracil

GI gastrointestinal

HCl hydrochloric acid

Hepes hydroxyethyl piperazineethanesulfonic acid

HIF1- α hypoxia-inducible factor 1- α

HIPEC hyperthermic intraperitoneal chemotherapy

HR homologous recombination

HRAS Harvey rat sarcoma virus

IHC immunohistochemical

IL interleukin

IORT intraoperative electron beam radiation

IR ionizing radiation KCl

potassium chloride ki-67 marker of

proliferation Ki-67 KRAS Kirsten

rat sarcoma virus

LAR low anterior resection

LARC locally advanced rectal cancer

LCRT long-course chemo-radiotherapy

LOH loss of heterozygosity

MAPK mitogen-activated protein kinase

MERCURY Magnetic Resonance Imaging in Rectal Cancer European Equivalence Study miRs microRNAS

MLH1 MutL homolog 1, colon cancer, nonpolyposis type 2

MMP-2 metalloproteinase-2

MMP-9 matrix metalloproteinase-9

MMR mismatch repair

MRC Medical Research Council MRI

magnetic resonance imaging mRNA

messenger RNA

MSH2 DNA mismatch repair protein Msh2

MSI microsatellite instability

MTHFR 5,10-methylenetetrahydrofolate reductase nCRT

neoadjuvant chemo-radiotherapy

NHEJ non- homologous end joining

NHS National Health Service

NICE National Institute for Clinical Excellence

NRAS neuroblastoma RAS viral oncogene homolog

NT non-targeting

OPRA organ preservation in rectal adenocarcinoma

P27 cyclin-dependent kinase inhibitor 1B

P53 tumour protein P53

PARP poly (ADP- ribose) polymerase

PBS phosphate buffered saline PC

peritoneal carcinomatosis pCR

pathological complete response

PCV packed cell volume

PET positron emission tomography scanning

PJS Peutz–Jeghers Syndrome

PMS2 mismatch repair endonuclease PMS2

PMSF phenylmethylsulfonyl fluoride

RAS rat sarcoma virus

RCTs randomised control trials

RISC RNA induced silencing complex

RNA ribonucleic acid

RPMI Roswell Park Memorial Institute

RT radiotherapy

SCPRT short-course therapy

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

SIP sphingosine-1-phosphate siRNA small interfering RNA SK

sphingosine kinase

SMI small molecular inhibitor

SNP single nucleotide polypmorphism

SPH sphingosine

SSB single-strand break

STR short tandem repeats

TEM trans-anal endoscopic microsurgery approach

TEUS trans anal endoscopic ultrasound

TG Tris-glycine

TGS 1 % Tris-glycine SDS

TILs tumour-infiltrating lymphocytes

TMA tissue microarray

TME total mesorectal excision

TNF tumour necrosis factor

TNM tumour, node, metastasis

TOPO topoisomerase

TRG tumour regression grading systems

UV ultraviolet

VEGF vascular endothelial growth factor

WCE whole cell extract

Contents

Chapter 1 Introduction

1.1 Epidemiology of colorectal cancer

Colorectal Cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide, with 1.8 million new cases and almost 890,000 deaths estimated to have occurred in 2018. It second commonest cause of cancer death globally (*Colorectal cancer Source: Globocan 2018 Number of new cases in 2018, both sexes, all ages*, 2018). Rates are higher in males when compared to females worldwide.

Colorectal cancer is the fourth most common cancer in the UK after breast, prostate and lung accounting for 11% of all new cancer diagnoses (Cancer Research UK, 2017). There were over 42, 0000 new cases of colorectal cancer in the UK in 2017: 23,500 (56%) in men and 18, 600 (44%) in women. It is the third most common cancer in both males (13% of the male total) and females (11%) separately. Colorectal cancer is the second most common cause of cancerrelated mortality in the UK after lung cancer. Over the last decade, colorectal cancer incidence rates have increased overall by 5%. The increase is higher in females; where rates have increased by 6%, compared to 3% in males. In the UK, 1 in 14 men and 1 in 19 women will be diagnosed with bowel cancer during their lifetime (Cancer Research UK).

Rectal cancer accounts for the most frequent site of tumour location with 32% and 23% in males and females respectively as a percentage distribution of colorectal cancers in 2012. An average total of 13,900 cases of rectal cancer were diagnosed annually (2012-2014) with an average 6,300 cancer-specific deaths annually during the same period (Cancer Research UK and provided by the London School of Tropical Medicine).

1.2 Aetiology of colorectal cancer

Cancer itself is a spectrum of diseases involving genetic mutations within cells that leads to the uncontrolled proliferation of abnormal cells and a potential ability to invade and spread from

the primary site (metastasis). Colorectal cancer itself is a multifactorial disease with a complex aetiology of genetic, environmental (smoking, obesity, dietary factors) and inflammatory conditions of the gastrointestinal tract being implicated in the transformation of normal colonic epithelium into invasive cancer.

In order to understand the concept of a cell acquiring genetic mutations and in turn becoming cancerous the regulation of cellular replication must first be explained.

1.2.1 The cell cycle

All cells with complete sets of genetic material are the product of multiple divisions and replications and passing on all their genetic information onto their progeny is fundamental to life. In eukaryotic cells (containing a nucleus with their genetic material and membrane bound organelles), this replication is governed by the cell cycle, which is a series of events that leads to a cell replicating its deoxyribonucleic acid (DNA) and then dividing into two identical daughter cells. This process is different for prokaryotes (cells without nuclei, such as bacteria). For eukaryotes, the process involves three phases; interphase, mitosis (the replication chromosomes within a nucleus to form two identical copies) and cytokinesis (the splitting of the cell into two identical daughter cells). Cancer is a spectrum of disease which culminates in the rapid and un-controlled division and replication of abnormal cells as a result of dysregulation of this cell-cycle.

1.2.2 Cell cycle control

Throughout the cell cycle there exists “check points” that allow the cell to ensure that it can proceed into the next phase and also identify any issues such as DNA damage then allow subsequent pause for repair. It is the loss of the normal check points within the cell cycle that can allow a cell to progress through the cell-cycle incorrectly allowing for unregulated proliferation and cells with unrepaired DNA damage. This is an important step in oncogenesis

and the transformation of normal tissue into neoplastic disease because cells may progress with DNA damage and replicate therefore compromising the integrity of the genome, this is known as "chromosomal instability". Cyclin dependant kinases (CDKs) and cyclins are important proteins in controlling the check points and determining if the cell progresses from G1 to S phase and G2 to M phase.

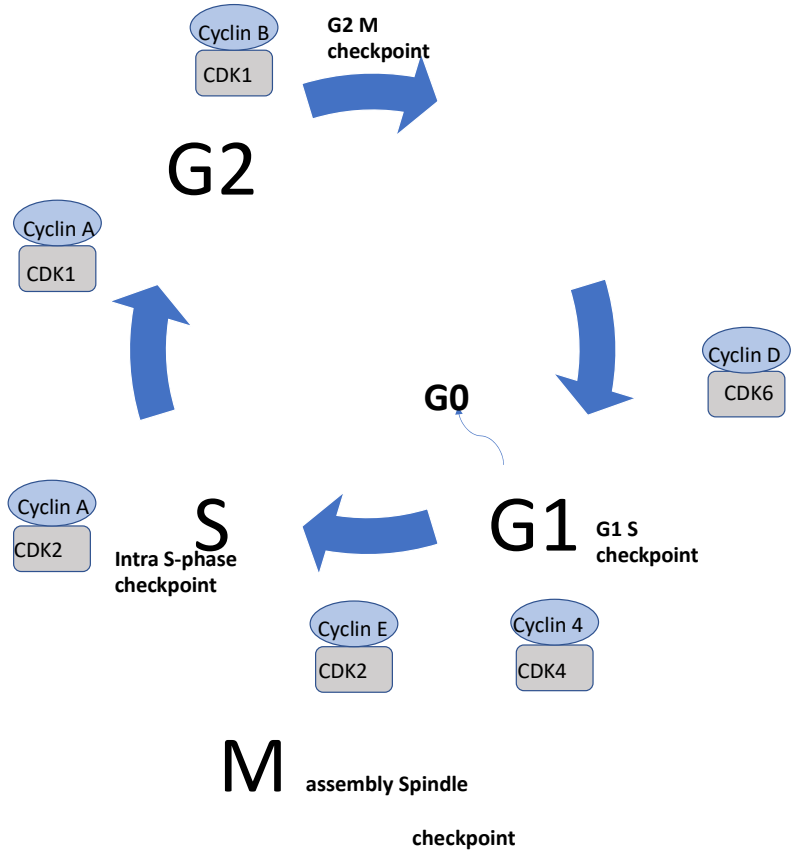


Figure 1.1 – *The Cell Cycle* – Diagram to illustrate the cell cycle and the role of cyclin and cyclin dependent kinases (CDKs) on cell cycle progression. M is the "mitosis" phase, i.e. in eukaryotic cells where the parent cell divides into two identical daughter cells. S is the interphase between the G or "gap phases", this is where the cell grows and prepares for mitosis.

The check points are crucial in determining whether the cell is in a healthy position to progresses through the cell-cycle or undergo an alternate fate such as cell death or cell-cycle arrest.

This tight regulation of the cyclin-cdk complexes is critical since it is their catalytic activity that subsequently determines the phosphorylation status of other proteins important in the regulation of the cell cycle (Tannoch, Hinds and Tsai, 2000). P27 and p53 are other important proteins in regulating progression through the cell cycle. P53 is perhaps the most important overall in terms of cell cycle regulation and is ultimately responsible for either halting cell cycle progression or inducing programmed cell death (apoptosis) in response to DNA damage.

1.2.3 The molecular pathogenesis of colorectal cancer

A key model that was first described by Vogelstein et al. in 1990 is the "Adenoma – Carcinoma sequence" model of tumourigenesis. This is the pathway involving multiple mutations within a population of initially normal colon cells that first develop into an adenomatous polyp (a proliferation of epithelial tissue projecting from a mucous membrane) into a dysplastic polyp (abnormal cells with the potential to become malignant) and eventually a colonic carcinoma (Figure 1). Genetic mutations occur in a multistep mechanism in several characteristic genes, such as loss-of-function in adenomatous polyposis coli (APC) gene (5q) which occurs in the early stage of adenoma formation, gain-of-function of Kirsten rat sarcoma (KRAS) oncogene (12p12) which occurs in the progression to large adenoma, and loss-of-function of tumour suppressor genes TP53 (17p) and netrin receptor DCC (DCC) (18q) for adenoma to adenocarcinoma progression (Fearon and Vogelstein, 1990). Loss of heterozygosity (LOH) can occur in metastatic lesions on 10q or gains of DNA sequences at 5p and 6p (Muñoz-Bellvis *et al.*, 2012). The most common histological subtype of CRC is adenocarcinoma but can also

include, mucinous carcinoma, adenosquamous carcinoma, signet-cell carcinoma and medullary.

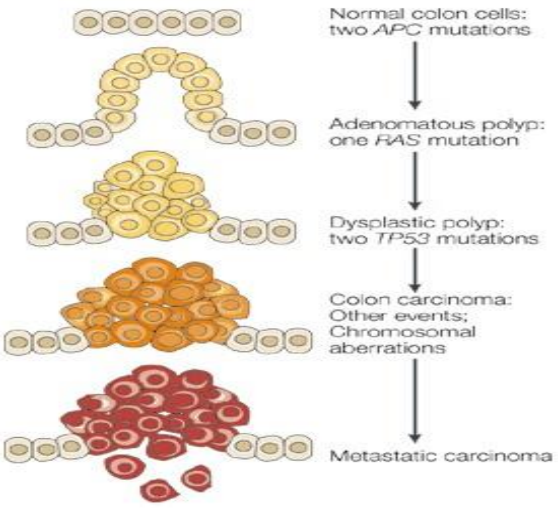


Figure 1.2 ***The transformation from normal colonic tissue into carcinoma.*** Diagram to illustrate the transformation of normal colonic epithelium into an adenomatous polyp then a dysplastic polyp and finally onto metastatic carcinoma. (Taken from Knudson, 2001).

1.2.4 Genetics of colorectal cancer

There are three different modes of presentation with regards to CRC, these are sporadic, inherited and familial. The majority (65%) of CRC occurs through sporadic genetic and epigenetic mutations within the patient genome with approximately 35% being due to genetic inheritance (Burt, 2007). Initially it was postulated that genetic and epigenetic mechanisms were distinct entities, however more recently it has been found that there is a complex interplay between genetic and epigenetic alterations in CRC pathogenesis (Coppedè *et al.*, 2014).

Genomic instability results in either point mutations or as chromosomal re-arrangement and is a characteristic of CRC pathogenesis. Genomic instability is further divided into chromosomal instability (CIN) or microsatellite instability (MSI). CIN has been found in around 85% of CRC with MSI in the remaining 15% (Dunican *et al.*, 2002).

CIN is characterised by aneuploidy (abnormal numbers of chromosomes within cells) and LOH. CIN has been shown to confer poorer responses in CRC and therefore in conjunction with MSI could serve as a prognostic marker (Walther, Houlston and Tomlinson, 2008).

MSI is the other subtype of genomic instability that is characterised by mutational alteration of simple repetitive sequences (expansions or contractions) that result in a frameshift mutation (Coleman and Tsongalis, 2006). Lynch syndrome accounts for about 3.3% of colorectal tumours leading to over 1,100 colorectal cancers a year in the UK, it is the most common inherited CRC syndrome. An estimated 175,000 people in the UK have Lynch syndrome. It is caused by genetic mutations in DNA mismatch repair (MMR) genes including mutL homolog 1, colon cancer, nonpolyposis type 2 (MLH1) , DNA mismatch repair protein Msh2 (MSH2), mutS homolog 6 (MSH6), and mismatch repair endonuclease PMS2 (PMS2). It can also be caused by mutations in a non-MMR gene, epithelial cell adhesion molecule (EPCAM). The loss of MMR protein results in genomic instability with numerous genetic alterations frequently found in non-encoding microsatellite regions (Iino *et al.*, 2000). The National Institute for Health and Care Excellence (NICE) has published recommendations for all patients presenting with CRC in February 2017 to be screened for MSI to identify patients with Lynch Syndrome as there is a strong association with other cancers such as ovarian, breast, endometrial and gastric cancer. MSI can be split into MSI-H and MSI-L, which confers high or low risk mutations respectively. Around 15% of sporadic CRCs are found to have MSI-H mutations and these are often in hypermutated subtypes.

1.2.5 Important genes and epigenetic markers in CRC

Kirsten rat sarcoma viral oncogene (KRAS)

The KRAS proto-oncogenes are important specifically in CRC. Rat sarcoma virus (RAS) proto-oncogenes Harvey rat sarcoma virus (HRAS) , KRAS , and neuroblastoma RAS viral oncogene homolog (NRAS) are key regulators of intracellular signalling pathways (Irahara *et al.*, 2010).

KRAS is the most common form of RAS that is mutated and associated with human cancer, and specifically in CRC approximately 35-50% of tumours have KRAS mutations (Lee *et al.*, 2017). Crucially it is the combination of KRAS and APC mutations that causes adenoma to progress to cancer (Bazan *et al.*, 2005). KRAS mutation and cancer phenotype in CRC remains unclear with different codons being associated with different tumour site and codon 12 KRAS mutations specifically being associated with poor survival rates in advanced disease (Jones *et al.*, 2017).

v-raf murine sarcoma viral oncogene homolog B1 (BRAF)

BRAF is a downstream effector molecule of KRAS in the mitogen activated protein kinase (MAPK) pathway, and BRAF mutations are present in 5-22% of CRCs with the V600E being the most common and found in MSI-H cancers. Some studies have shown that BRAF mutations are associated with more aggressive CRC and poorer survival, however other studies suggest it may the MSI status that acts as a confounder (Coleman and Tsongalis, 2016).

Tumour protein 53 (TP53)

TP53 is well known as a cell cycle checkpoint regulator and plays a key role in determining cell fate through either survival by DNA damage repair, or cell death through apoptosis. It is associated with the progression from adenoma to invasive cancer. Loss of function of the TP53 gene and also gain of function promotes tumour progression and invasion by alternating tumour metabolomics (Liu *et al.*, 2015).

1.2.6 Epigenetics in CRC

Epigenetics is where gene function is altered without DNA bases being changed, and this can be mediated through DNA methylation, ribonucleic acid (RNA) modification and histone modifications. Its role in cancer is evolving and this is particularly relevant in CRC. CpG islands are regions of the genome that are 200 base pairs long with over 50% composition of

cytosine and guanine. They are typically found in or near 50% of promoter gene regions, and therefore play an important role in controlling gene expression (Brenner, Miller and Broughton, 2002). The DNA within these 5'—C—phosphate—G—3' (CpG) islands can be methylated which in turn affects how certain genes are expressed, in general, methylation leads to reduced gene expression. CpG island hypermethylation phenotype is known as CpG island methylator phenotype (CIMP) and if these occur within promoter regions of tumour suppressor genes, then it will lead to loss of function of that gene and therefore increased cell proliferation and cell cycle progression. It has been shown that CIMP positive CRCs develop via a serrated pathway (East *et al.*, 2017).

1.2.7 Familial colorectal cancer syndromes

Inherited colorectal cancer syndromes are rarer than sporadic CRC accounting for approximately 2-5%. These inherited syndromes are broadly split into Lynch syndrome and various polyposis syndromes that are then further subdivided into the polyp subtype that is predominantly found.

Familial adenomatous polyposis (FAP) is the second most common inherited CRC syndrome caused by various mutations APC gene on chromosome 5q21 in CRC (Kinzler and Vogelstein, 1996). It accounts for approximately 1% of all CRCs (Mulvihill, 1983). This syndrome is characterised by innumerate adenomatous polyps within the colon. Half of all patients will develop polyps by the age of 15 and almost all patients have polyps by 35 years old (Petersen, Slack and Nakamura, 1991).

Peutz–Jeghers Syndrome (PJS) is an autosomal dominant inherited gastrointestinal polyposis syndrome associated with mucocutaneous hyperpigmentation. The most consistent features of PJS are mucocutaneous melanin hyperpigmentation, hamartomatous polyps (non-neoplastic consisting mainly of connective tissue) and a family history of PJS. PJS confers a 90% risk of cancer development, with 70% risk of CRC and 50% for breast (Jansen *et al.*, 2006) (Dunlop,

2002). In contrast to other polyposis syndromes, the polyps are mainly located in the small bowel with a lower proportion in the stomach and colon (Brosens *et al.*, 2015). The exact mechanisms of how the polyps develop and how they turn into cancer is still not fully elucidated. However stem cell analysis has shown time for cells to expand and populate a crypt is longer therefore more time for accumulation of mutations to occur (Langeveld *et al.*, 2012).

1.2.8 Microbiome in CRC

The relatively low heritability of CRC implies that environmental factors play a key role in the pathogenesis. Among environmental factors, the biology of the microorganisms within the human body – the "microbiome" has become a key feature of cancer. The microbiome has been implicated in several cancers, including CRC. The colon and rectum hosts approximately 3×10^{13} bacteria with a complex and dynamic real-time interaction between the microbiome and the epithelium (Qin *et al.*, 2010). These interactions include metabolism, inflammatory changes within the gastrointestinal epithelium and immunomodulatory changes.

Early mouse studies showed that certain gastrointestinal bacteria played a role in promoting carcinogenesis through aberrant crypt formation in gut epithelium when exposed to the carcinogen 1,2-dimethylhydrazine (Onoue *et al.*, 1997). Human studies have taken a metagenomic approach and identified that the CRC microbiome is fundamentally different to the microbiome of healthy individuals in terms of the density, variety and species of bacteria present. This supports functional evidence for the importance of the composition of the gut microbiome and that certain species of bacteria may be pro-carcinogenic (*Bacteroides, Escherichia, Fusobacterium*, *Porphyromonas)* and others are relatively protective (*Roseburi*a) (Repass *et al.*, 2018) (Feng *et al.*, 2015) (Yu *et al.*, 2017).

The transformation of adenoma to carcinoma in CRC is a key process in pathophysiology and recent microbiome studies have shown that in patients with colorectal adenomas or very early cancers, they exhibit higher proportions of certain bacteria e.g. Fusobacterium (McCoy *et al.*,

2013). Different bacteria and also fungi were however prevalent in different concentrations at disease specific points along the adenoma – carcinoma pathway (Yachida *et al.*, 2019) (Luan *et al.*, 2015). These findings are promising as current faecal current stool- based tests cannot identify the risk of malignant transformation in early colorectal adenomas.

1.3 Screening for CRC

The "NHS Bowel Cancer Screening Programme" was introduced in 2006 initially inviting those from 60-69 years old to complete 2-yearly faecal occult blood testing (FOB), however, this has now been extended to 74 years. The FOB test measures the presence of occult blood within the stool from three separate bowel movements on guaiac paper that reacts positively to haem when exposed to hydrogen peroxide. If positive, then patients are invited for endoscopic investigation. Since June 2019, the standard FOB kit has been replaced with the faecal immunochemical test (FIT) test. The FIT test provides quantitative immunohistochemical analysis of occult blood level present within the stool sample and also only requires one sample to be sent for analysis. At present, the FIT test has a sensitivity of 79% for detecting CRC and 25–27% for detecting advanced colorectal adenomas (Lee *et al.*, 2014) (Hundt, Haug and Brenner, 2009).

Since 2011, an additional "Bowel Scope" has also been implemented where at the age of 55 years old, patients are invited to undergo a single flexible sigmoidoscopy. The Cochrane Database of Systematic Reviews (2006) concluded that this screening programme has resulted in a 16% decrease in overall mortality from colorectal cancer in all patients screened.

1.4 Diagnosis

The majority of patients diagnosed with CRC present based on presentation with symptoms particularly with lower gastrointestinal (GI) bleeding (haematochezia) as a predominant feature in rectal cancer. Patients can also present as an emergency, and acutely unwell with signs

suggestive of bowel obstruction with or without evidence of intrabdominal sepsis. Although a significant proportion of patients are diagnosed routinely through screening who may be asymptomatic, it is still symptomatic presentations that lead to a higher diagnosis rate in CRC. Histology is obtained at endoscopy; CRC is mostly adenocarcinoma (> 90%), however it can also include neuroendocrine, squamous cell, adenosquamous, spindle cell, lymphoma, carcinoid, sarcoma and undifferentiated carcinomas. Adenocarcinoma is characterized by glandular formation and this forms the basis of tumour grading scores:

1. Well differentiated adenocarcinoma >95% of the tumour is gland forming.
2. Moderately differentiated adenocarcinoma shows 50-95% gland formation.
3. Poorly differentiated adenocarcinoma is mostly solid with <50% gland formation.

Most colorectal adenocarcinomas (~70%) are diagnosed as moderately differentiated with well and poorly differentiated carcinomas account for 10% and 20%, respectively. Immunohistochemistry also plays a key role in diagnosis and cytokeratin (CK) 20, CK7 and homeobox protein CDX2 (CDX2) are the most widely used immunohistochemical markers used in CRC diagnosis. Positivity for CK20 and negativity for CK7 is the most common immunohistochemical pattern in cancer of colorectal origin (Fleming *et al.*, 2012).

As described earlier, MSI status has now also become an established tool in diagnosis in addition to RAS, NRAS and BRAF mutational analysis as this will have a bearing on specific treatment options available.

1.5 Rectal cancer staging

1.5.1 Radiological staging

Radiological imaging is crucial to correctly locate the tumour, describe the presence of any local invasion and determine if there is any distal metastatic disease. It is these factors that

determine if patients are considered for any pre-operative treatment (chemotherapy, radiotherapy or combination chemo-radiotherapy (CRT)), offered surgery as a first line treatment or treated conservatively.

The TNM (Tumour, node, metastasis) staging system from the American Joint Committee on Cancer (AJCC) (2017 edition) is the gold standard system used for pre-operative staging in CRC. Tumours are staged with the prefix of "c" TNM if this is pre-operative and "p" illustrates complete pathology results following oncological resection, the letter "y" denotes any specimen that has been obtained where the patient has undergone pre-operative (neoadjuvant) therapy.

The TNM system (Table 1) will comprise of results gained from several radiological investigations:

1. Computerised tomography (CT) scanning of the chest, abdomen and pelvis will demonstrate regional tumour involvement, lymph node morphology, distant metastatic disease and any specific complications of the tumour (abscess formation, fistulating disease or malignant obstruction). CT is the modality of choice for determining metastatic burden (M). Triple-phase contrast magnetic resonance imaging (MRI) of the liver can be arranged for accurate characterisation of liver lesions.

2. The most suitable imaging modality for accurate staging (T,N) of rectal cancer is T2-weighted MRI imaging of the rectum and pelvis, as it provides information on the level of tumour invasion through the bowel wall, detailed lymph node morphology and distance between the tumour and the circumferential resection margin (CRM). The CRM is the optimal resection distance around the tumour in cross-section that contains the mesorectum, which allows the maximal oncological benefit from surgical resection in the form of a total mesorectal excision (TME). The Magnetic Resonance Imaging in Rectal Cancer European Equivalence Study (MERCURY) trial showed if the distance

between tumour and CRM was < 1mm, this was associated with higher rates of locoregional recurrence following surgery (Brown, 2006). As a result of this evidence, patients with threatened margins are offered neoadjuvant CRT in an attempt to decrease the size of the tumour (down-staging).

3. Trans anal endoscopic ultrasound (TEUS) is an alternative method to evaluate rectal tumours and can be useful in characterising T1/T2 tumours particularly where MRI may be contraindicated (patients who have cardiac pacemakers or retained metallic material) and where there is specific consideration for endoscopic removal of a rectal tumour (Gao *et al.*, 2020). The consensus guidelines state that MRI is still superior in the preoperative staging setting (Glynne-Jones and Brown, 2017) and remains the gold-standard.
4. Positron emission tomography scanning (PET) employs administration of a radiopharmaceutical (specifically Fluorine-18) added to glucose which will be taken up by highly metabolically active tissue and tumour cells, the patient is then imaged using a gamma camera to produce a map of areas of high uptake. It can help clarify whether areas of radiological suspicion (i.e. enlarged lymph nodes or extra-gastrointestinal deposits) show increased uptake therefore reflecting malignant involvement.

	T Staging
T1	*Tumour invades mucosa and submucosa*
T2	*Tumour invades but does not penetrate muscularis propria*
T3	*Tumour invades subserosa through muscularis propria* *T3a: tumour extends < 1mm beyond muscularis propria* *T3b: tumour extends > 1-5mm beyond muscularis propria* *T3c: tumour extends > 5-15mm beyond muscularis propria T3d: tumour extends >15mm beyond muscularis propria*
T4	*Tumour invades peritoneal reflection (T4a) or adjacent organs (T4b)*

	N Staging
N0	*No metastatic lymph nodes*
N1	*Metastases in 1-3 perirectal nodes*
N2	*Metastases in 4 or more perirectal nodes*
	M Staging
M0	*No distant metastases*
M1	*Distant metastases*

Table 1.1- *The TNM pre-operative (radiological) staging system (adapted from the AJCC (2017))*

1.5.2 Pathological staging

The Duke's classification was originally developed as pathological staging of a colorectal cancer (Table 2) therefore can only be described once it has been surgically resected unlike the TNM system which is predominantly pre-operative and then modified following completed histology results from the resected specimen. Although Duke's classification is commonly described, it its important to note that it was not specifically designed to describe rectal cancer.

Dukes Classification

Dukes A: Invasion into but not through the bowel wall
Dukes B: Invasion through the bowel wall penetrating the muscle layer but not involving lymph nodes
Dukes C: Involvement of lymph nodes
Dukes D: Widespread metastases

Table 1.2 – *The Dukes pathological classification of CRC.*

This classification was then further modified by Astler and Coller in 1954 to subdivide Dukes B and C depending on invasion through the muscularis propria (muscle wall) of the colon.

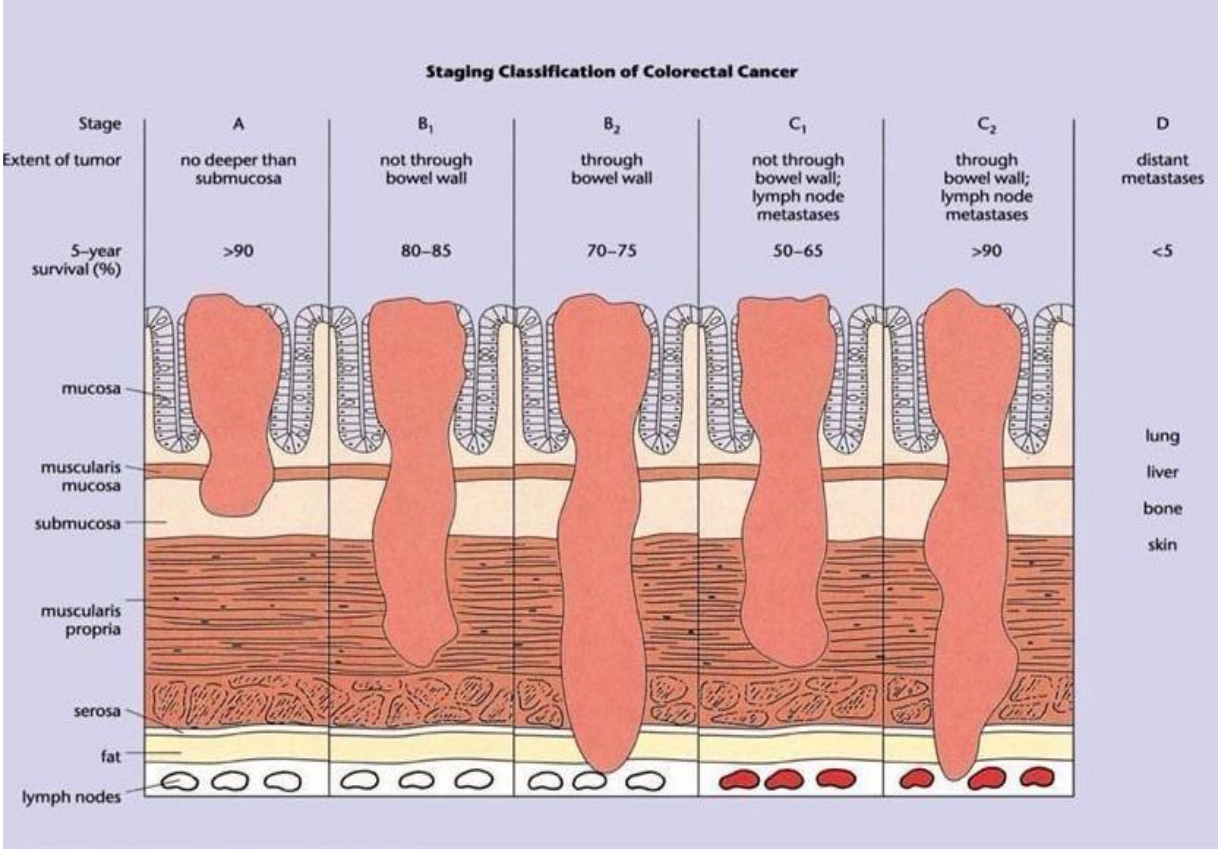


Figure 1.3 - *"Modified Dukes Staging of Colorectal Cancer".* (Taken from Atlas of Diagnostic Oncology 4th Edition, Arthur Skarin (2009)

1.6 Prognosis

In the UK, approximately 50-64% of rectal cancer is diagnosed as locally-invasive (Cancer Research UK), which if untreated leads to a five-year survival of < 5% (Solum, Riffenburgh and Johnstone, 2004). The prognosis for rectal cancer in terms of overall survival depends on the extend of the disease based on the TNM staging in conjunction with the other factors that have been described such as the sub-type of the tumour, vascular invasion, lymphatic invasion and distance to the CRM.

The TNM staging system can then be broadly classified into various stages (I to IV). Stage I meaning the tumour is within the muscularis propria (T1/T2, N0, M0), Stage II means the tumour can invade through the serosa but no lymph node involvement (T1-4, N0, M0), Stage

III means any T (T1-4, N1/2, M0) stage but the tumour has to involve regional lymph nodes and Stage IV is any T or N with distant metastatic spread.

The overall 5-year survival rate following surgery for rectal cancer are as follows: Stage I; 85100%, Stage II; 60-80%, Stage III; 30-50% and Stage IV: 14% (*The ASCRS Textbook of Colon and Rectal Surgery*, 2011).

1.7 Rectal cancer treatment

1.7.1 Surgical treatment

Surgery provides the highest probability of curative treatment, although the particular operation will depend on the stage TNM stage of the tumour, the size and its anatomical location within the rectum. There are locally resectable options available, which include Trans anal Excision (TAE) and the trans-anal endoscopic microsurgery approach (TEM). Both of these approaches are only suitable for superficial tumours (T0/1) that are less than 3cm in diameter.

Most patients present with more invasive disease which is not amenable to a local excision and will require more invasive surgery as either as a sphincter-sparing (preservation of the muscle fibres that maintain faecal continence), low anterior resection (LAR – involves removing the rectum with surrounding blood supply and lymph nodes) for tumours in the upper and middle third of the rectum and possible de-functioning ileostomy (small bowel brought to skin surface temporarily to allow for the resected bowel to heal distally), or a more extensive abdominal perineal resection (APR) for tumours in the distal third (<6cm from the anal verge) of the rectum.

LAR is the most common procedure employed for the treatment of rectal cancer. In 1986, RJ Heald described the Total Mesorectal Excision procedure (TME), that involves removing all the peri-rectal connective tissue, which when combined with LAR significantly reduced local recurrence rates and improved overall survival as shown by the Dutch TME trial (Kapiteijn, Putter and Van De Velde, 2002). The importance of adhering to the correct surgical planes to

achieve a complete TME and its associated oncological benefits were quantified using a grading system for TME resection specimens by Quirke in 2009 (Quirke *et al.*, 2009). As such, it has become the gold standard method for surgical therapy in rectal cancer. LAR can be performed as an open surgical technique, laparoscopic technique and more recently as a robotic technique. Specifically with regards to open vs laparoscopic surgical techniques, the ALaCaRT trial showed that noninferiority of laparoscopic surgery compared with open surgery was not established (Stevenson *et al.*, 2015). The ROLARR trial did not show that a robotic approach was superior to a standard laparoscopic approach (Jayne *et al.*, 2017) and both laparoscopic and open techniques have shown the same oncological outcomes (Vennix *et al.*, 2014) .

1.7.2 Advanced surgical treatment options

Pelvic exenteration

Following curative surgery, the rate of local recurrence in rectal cancer can still be as high as 30% and although radiotherapy and chemotherapy play important roles, there exists a need for advanced surgical resection options (Sagar and Pemberton, 1996).

Pelvic exenteration is a complex surgical procedure that involves removing multiple organ structures within the pelvis and was initially described as a palliative procedure for advanced cervical cancer (Brunschwig, 1948). This surgery can be performed as a primary procedure to remove the rectal cancer where it has either invaded anteriorly into the urinary or reproductive organs of the pelvis or posteriorly towards the sacrum. It can also be performed as "salvage" surgery following recurrence of disease after initial primary resection. Understandably, pelvic exenteration carries a significant risk of morbidity but the outcomes have improved over time as technique and patient selection have been refined (Lopez, Standiford and Skibba, 1994).

Liver metastatic disease

Around 13% of patients with rectal cancer can present with synchronous (tumour found at the same time or prior to rectal primary) liver metastases (Tan and Ooi, 2010), although this

number increases to approximately 30% in locally advanced rectal cancer (LARC) (Viganò *et al.*, 2011). Untreated colorectal liver metastases convey a poor prognosis and hepatectomy is the only potential curative option with 5-year survival rates of 26-50% (Pavlidis, 2011). Unfortunately only 10-30% of patients with synchronous liver metastases are suitable for liver resection (Minagawa *et al.*, 2006). If deemed suitable at multi-disciplinary discussion, patients can be offered a simultaneous resection of the primary rectal cancer and the liver metastases or a staged resection. There is currently no definitive evidence to support any of these approaches in terms of surgical or survival advantage (Kelly *et al.*, 2015).

Peritoneal carcinomatosis

Cytoreductive surgery (removing as much tissue as possible from the abdomen including the peritoneum) with hyperthermic intraperitoneal chemotherapy (HIPEC) has become established in treating peritoneal carcinomatosis (PC) in CRC. Prospective randomized trial data with 8 years of follow-up demonstrated a 45 % 5-year survival in patients with PC from CRC (Verwaal *et al.*, 2008). Genetically, colon cancer behaves differently to rectal cancer and this is now well-established in the literature. It has been shown that rectal cancer accounts for lower incidence of PC compared to colon cancer and therefore lower rates of treatment with HIPEC and cytoreductive surgery (Elias, 2010). However, 3-year survival for both colon and rectal cancer patients is comparable (Votanopoulos *et al.*, 2013).

1.8 Radiotherapy

1.81 Principles of radiotherapy

The use of ionizing radiation (IR) for medical therapy has evolved into a distinct clinical specialty. IR can be broadly split into two groups; “photon radiation” (x-rays and gamma rays) or “particle radiation” (e.g. protons and alpha particles). Radiotherapy utilizes high-energy electromagnetic and particle radiation to treat malignant tumours predominantly by causing DNA damage within cancer cells. The main limitation of radiotherapy is the balance between

radioresistance of the tumour cells and collateral damage to the surrounding healthy tissue. The issue of collateral damage to surrounding healthy tissue was identified by Coutard in the 1900s, who developed the concept of "fractionated therapy" as opposed to administering single large radiation doses which caused significant tissue injury.

1.8.2 Modes of radiotherapy delivery

All forms of radiotherapy will generate IR that will damage cellular DNA within malignant cells. Radiotherapy itself can either be applied externally (external beam), internally placed within the body (brachytherapy) or systemically by administering radiopharmaceuticals (Iodine-131).

For patients where the resections margins are involved for advanced locally invasive rectal cancer then intraoperative or where there is low volume inoperable residual disease, then intraoperative electron beam radiation (IORT) can be given.

"Papillon" low dose contact brachytherapy is a specific example of using low dose x-ray radiation applied directly to rectal tumours (<3cm in size and superficial) via a tube inserted through the anus and into the rectal lumen. This technique has been used routinely in clinical practice at the Clatterbridge Cancer Centre (Wirral, United Kingdom) since 1993.

1.8 3 Mechanisms of action of radiotherapy

Radiotherapy targets intracellular DNA and the various different forms of IR have different ionization densities of the paths in which they travel. IR may pass through the cells and ionize DNA directly (direct action) or ionize water molecules producing highly volatile OH radicals, which can then indirectly ionize DNA (indirect action). DNA damage secondary to IR can result in base damage or disruption of the sugar-phosphate backbone which can ultimately lead to a single or double-strand break in the helix (SSB/DSB) or a DNA crosslink. For example, a dose of 1 Gy of radiation has been estimated to cause approximately 3000 damaged bases, 1000

SSBs and 40 DSBs (Hall and Giaccia, 2012). For most cells, base damage and SSB are effectively repaired by the base excision repair system (BER) (Grundy and Parsons, 2020) (Carter and Parsons, 2016) It is the DSBs, particularly caused by radiation that are the most complex to repair (Vitti and Parsons, 2019). Cells repair DSBs through the defined pathways of non- homologous end joining (NHEJ and homologous recombination (HR) (Scully *et al.*, 2019).

1.8.4 Radiotherapy and apoptosis

Apoptosis is a highly regulated energy-dependant process where a cell will undergo a programmed cell death following a variety of stimuli, including radiotherapy. Radiation therapy will mostly cause the intrinsic (mitochondrial damage) apoptotic pathway, however depending on the dose administered and the cancer cell type, the extrinsic pathway can also become activated (Takasawa *et al.*, 2005). If a cell is unable to repair SSBs and DSBs following radiation-induced damage then this will signal the activation of the intrinsic pathway (Gudkov and Komarova, 2003). Due to the nature of cancer cells rapidly dividing with a higher incidence of faulty DNA repair pathways it will make them intrinsically more susceptible to irradiation when compared to healthy tissues. The method in which the cells undergo death following irradiation depends on a variety of factors, including the cell type along with its phase in the cell-cycle, the oxygen available and the quality of the irradiation (Stewart *et al.*, 2011).

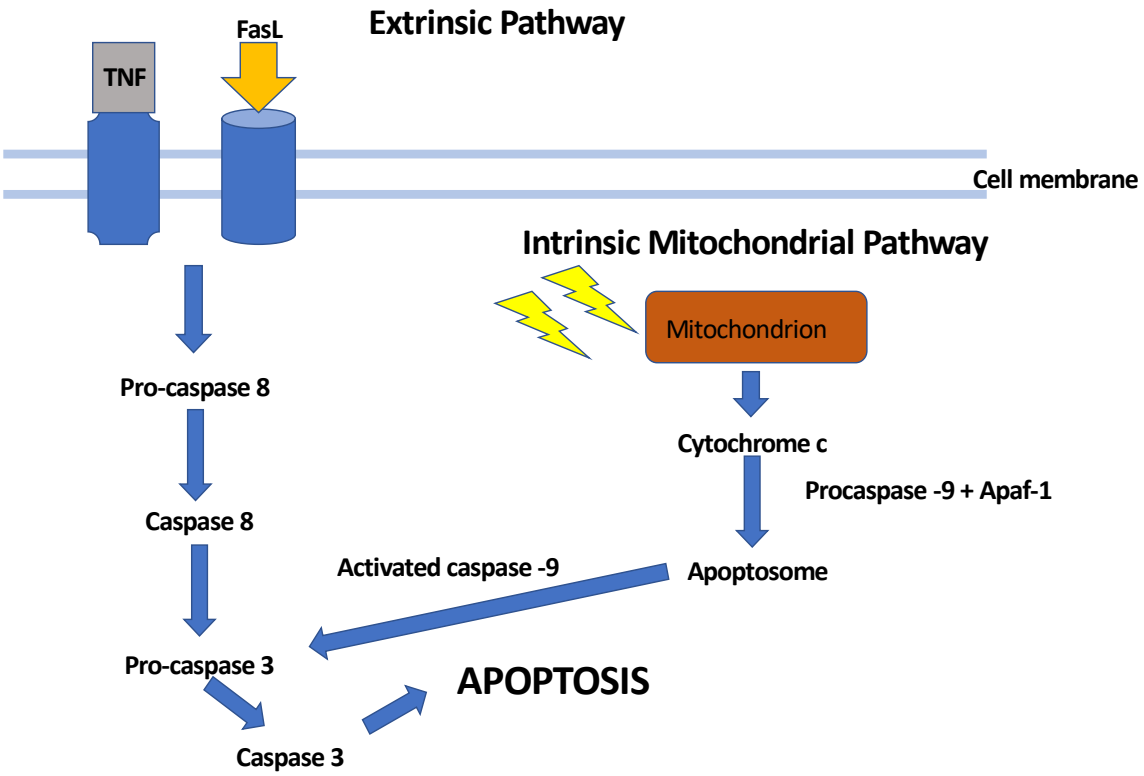


Figure 1.4 - ***The Apoptotic Pathway***. IR mainly affects the intrinsic pathway leading ultimately to Caspase 3 activation and apoptosis. (Adapted from Bhosale *et al.*, 2020)

1.9 Radiotherapy treatment regimens

Surgery is only one consideration for treatment of locally advanced rectal cancer (LARC) and there a need for additional treatments to reduce the risk of local recurrence (Marsh, James and Schofield, 1995). Radiotherapy (RT) and chemoradiotherapy (CRT (RT + a chemotherapy agent as a radiosensitiser)) are crucial methods of treatment in LARC to reduce the risk of local recurrence.

1.9.1 Neoadjuvant treatment

Pre-operative (neoadjuvant) pelvic radiotherapy was found to be successful in reducing rates of recurrence from approximately 25% (which was a significantly higher recurrence rate

compared to most centres) in the Swedish Rectal Cancer Trial (Påhlman, 1997) to around 5-10% (Sebag-Montefiore *et al.*, 2009), and there is evidence for both pre and postoperative radiotherapy in the reduction of recurrence disease in rectal cancer (Colorectal Cancer Collaborative Group, 2001). Neoadjuvant radiotherapy has also been shown to reduce the size of the tumour (downstaging) by around 16% (Bosset *et al.*, 2016) which can facilitate surgical resection through affecting the distance between tumour and CRM or sphincter complex therefore providing surgical options that have reduced associated morbidity (Graf *et al.*, 1997).

At present, there are two main methods for neoadjuvant radiotherapy. Short-course therapy (SCPRT) and long-course chemo-radiotherapy (LCRT). SCPRT involves delivering a total of 25Gy in total of radiation to the tumour delivered in 5Gy doses over 5 days (Sebag-Montefiore *et al.*, 2009) and surgery within a week. LCRT varies from SPCRT as it also includes a fluoropyrimidine such as 5-fluorouracil (5 FU) as a drug to make the tumour cells more sensitive to radiotherapy (radiosensitiser). LCRT typically involves delivering 50.4Gy total dose in 1.8Gy divided doses over approximately 5 weeks with a period of 8-10 weeks rest prior to surgery (Bosset *et al.*, 2006). Both LCRT are comparable in terms of reduction of recurrent disease, survival and toxicity however there may be evidence to suggest that LCRT reduces local recurrence risk specifically in distal rectal tumours (Ngan *et al.*, 2012).

The compelling evidence for improved outcomes following neoadjuvant radiotherapy has come from several landmark trials:

1. The Swedish Rectal Cancer Trial (1997) (n=1168 short course RTx + surgery *vs* surgery alone) was the only one which has also shown improved survival although it is important to note their baseline recurrence rate was higher than comparable institutions.

2. The Dutch TME trial (2001) (n=1861 short course RTx + TME *vs.* TME alone)

3. The German CAO/ARO/AIO-94 trial (2004) (n = 823 patients preoperative CRT with fluorouracil (FU), TME, and adjuvant FU chemotherapy, or the same schedule of CRT

used postoperatively) that showed preoperative CRT was superior to postoperative treatment.

4. The Fédération Francophone de Cancérologie Digestive (FFCD) 9203 trial (2006) (n = 773 pre-operative chemoradiotherapy *vs* preoperative radiotherapy).
5. The Medical Research Council (MRC) CR07 trial (2009) (n=1350 short course RTx *vs* initial surgery with selective postoperative CRT).
6. European Organisation for Research and Treatment of Cancer (EORTC) 22921 trial (2014) (n = 1011) that showed for the first time that adjuvant (postoperative) radiotherapy showed no benefit when given after neoadjuvant therapy.

Data mainly from the Swedish trial highlighted the significant morbidity associated with radiotherapy including increased perineal and wound infections, increased ano-rectal disorders, increased late adverse events involving various organ systems and sexual dysfunction. This led to the development of standardized LCRT by the German CAO/ARO/AIO-94 Trial (2004).

Despite advances in neoadjuvant therapy in terms of reducing local recurrence the rate of distal metastatic disease remains around 30% and this is now the major cause of death in this cohort of patients (Peeters *et al.*, 2007). There is data for an increased role of neoadjuvant systemic chemotherapy to target micro metastatic disease therefore the significant risk of distant metastatic disease (standard practice is for adjuvant chemotherapy). The UK Phase II "Chemotherapy then Radiation then Immediate Curative Surgery for operable rectal cancer" (COPERNICUS) trial showed that preoperative chemotherapy showed promising tumour downstaging seen on MRI but further studies are needed (Gollins *et al.*, 2018a). Similarly, there is an impetus for developing total neoadjuvant chemotherapy strategies with all systemic chemotherapy being given prior to CRT and surgery with promising results in terms of recurrence and morbidity (Fernandez-Martos *et al.*, 2015).

At present, the most robust trial-based evidence is for neoadjuvant therapy in patients with T3/T4 disease. However it can also be considered in patients where tumour distance to CRM is < 1mm (a threatened margin), T1/2 lesions with lymph node involvement on imaging and distal tumours where there is a chance that down-staging can reduce an APR into a sphincter sparing procedure (Allal *et al.*, 2000).

1.9.2 Adjuvant therapy

In the United States, practice has been to administer post-operative (adjuvant) chemotherapy (5-FU based) for patients following rectal cancer resection in Stage II and III disease (Steele and Posner, 1993). The German trial (2004) demonstrated that neoadjuvant therapy was superior to adjuvant therapy with regards to local recurrence in LARC (6% local recurrence neoadjuvant arm *vs* 13% in the adjuvant arm). However, the American and German trials did not address the question of whether or not adjuvant therapy had any impact on survival. Currently, the NICE guidelines for patients with Stage III disease and high-risk (Glimelius *et al.*, 2013) Stage II rectal cancer is preoperative SCRT or neoadjuvant CRT followed by a TME then adjuvant chemotherapy is recommended, but the full benefits are still not entirely certain and it is uncertain if every patient with LARC requires adjuvant therapy (Bujko, Glynne-Jones and Bujko, 2010). A Cochrane review of 21 randomised control trials (RCTs) showed that adjuvant chemotherapy reduced risk of death and disease recurrence (Petersen *et al.*, 2012). Further work is needed to elucidate the benefits of adjuvant therapy in stage-specific subgroups in patients with LARC.

1.10 Radiosensitivity and radioresistance

DNA Damage Responses (DDRs)

The primary evolutionary goal of every organism is to successfully pass on in-tact genetic material to the next generation, this means that DNA has to be carefully protected from both

endogenous and exogenous threats. DDRs involve various complex sensing mechanisms to detect DNA damage and then perform various cellular responses including DNA damage repair. Each human cell sustains thousands of DNA damage lesions each day(Lindahl and Barnes, 2000). The most pervasive exogenous cause for DNA damage is ultraviolet (UV) light which can cause ~100,000 lesions per exposed cell per hour.

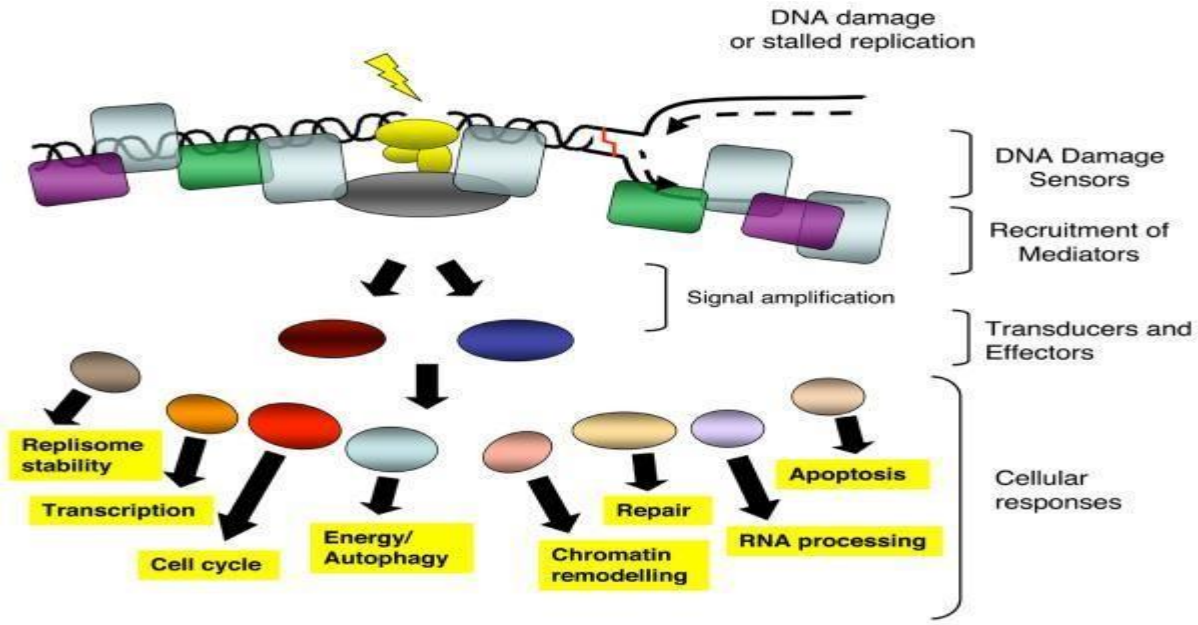


Figure 1.5 - ***The model for DDR.*** The presence of a lesion in the DNA, which can lead to replication stalling, is recognized by various sensor proteins. These sensors initiate signalling pathways that impact a wide variety of cellular processes. (Taken from Jackson and Bartek, 2009).

DNA repair

Ionising radiation tends to cause DSBs which are the most significant DNA-damage lesions that can be sustained. DSB break repair systems are vital for genomic integrity. Homologous recombination (HR) and Non-homologous end-joining (NHEJ) are the two main pathways involved in DSB repair. HR requires a sister chromatid as a template therefore can only occur in S and G2 phase of the cell-cycle, while NHEJ does not require a template and occurs

throughout the cell cycle. Both pathways are important in maintaining the stability of the genome.

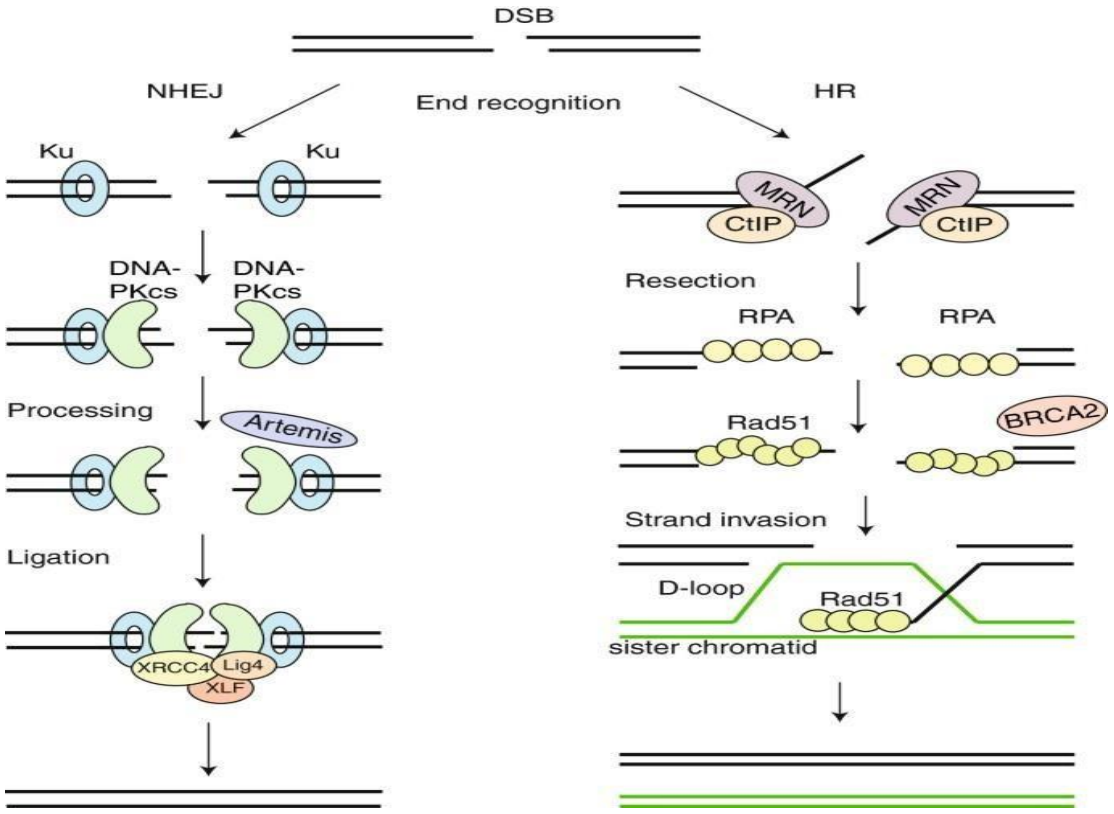


Figure 1.6 - ***Homologous recombination (HR) and Non-homologous end-joining (NHEJ)*** Diagram illustrates NHEJ and HR double strand break repairs and key enzymes involved. (Taken from Brandsma and van Gent, 2012).

1.10.1 Mechanisms of radioresistance

The cancer stem cell hypothesis is that within a tumour there is a population of "cancer stem cells" (CSC) that have the potential for self- renewal and infinite regeneration that drive tumour formation and can repopulate the tumour (Reya *et al.*, 2001). The aim of radiotherapy therefore is to eliminate all these CSCs. If a CSC survives treatment, then there is a potential for the tumour to recur.

There is also evidence that CSC are not only more radioresistant when compared to typical tumour cells, but can also obtain a more radioresistant phenotype when exposed to IR as an

evolutionary model of selection pressures conferring survival to resistant cells therefore changing the morphology of the tumour population. Increased ability for DNA repair mechanisms and reducing free-radical damage to DNA has been demonstrated in various cell lines when exposed to radiotherapy. Understanding these DDRs of tumour cells to DNA lesions allows for future potential therapeutic targeting (Desai, Yan and Gerson, 2018).

There are four key established factors that mediate chemo-radioresistance in tumour cells:

1. Larger tumours have higher proportions of hypoxic regions (reduced oxygen) therefore the necessary DNA damage caused by chemical radicals formed as a result of local ionization is not sufficient to kill all tumour cells (Steel and Peacock, 1989).
2. Cells in different phases of the cell cycle have different sensitivities to radiation, cells in proliferative phases have higher radiosensitivity (Zaider and Hanin, 2011).
3. Treatment refractive tumours display an increased ability to repair DNA damage and this can be IR-inducible (Weichselbaum, Dahlberg and Little, 1985)
4. Patients will invariably have different inherent genetic susceptibility to chemotherapy as higher grade drug induced organ toxicity can be a surrogate favourable prognostic marker of improved tumour response (Wolff *et al.*, 2011)

Further detailed mechanisms to elucidate observed phenotypes in rectal cancer and why certain tumours are more resistant has required high throughput analysis (whole genome) and low throughput studies (biomarkers).

Several groups have employed whole gene expression analysis in an attempt to identify genetic signatures associated with CRT resistance using biopsies from patients with LARC yet there is still no global consensus with regards to which specific gene signatures can predict CRT resistance phenotypes. This is in contrast to breast cancer, gene expression has proved more

translatable with 70 genes being validated as prognostic signatures that have been applied to a specific trial “Microarray in Node-Negative Disease May Avoid Chemotherapy” [MINDACT] trial) (S.C. *et al.*, 2012).

1.10.2 Radiosensitizers

Routinely, radiosensitizers are given with RT to enhance this either through causing additional DNA damage or through interference with the tumour cells’ ability to repair DNA damage. In clinical practice the most widely used is a single agent fluoropyrimidine concurrently with RT.

Fluorouracil

5-Fluorouracil (5-FU) is an antimetabolite fluoropyrimidine. It is widely used as a chemotherapeutic agent, particularly in colon and breast cancer. In colon cancer treatment it was the first radiosensitizer that was utilised. 5-FU exerts its cytotoxic effects through misincorporation of fluoronucleotides into RNA and DNA, and inhibition of thymidylate synthase which is a nucleotide synthetic enzyme (Longley, Harkin and Johnston, 2003).

There are several mechanisms thought to mediate how 5-FU acts as a radiosensitizer. Firstly, it is thought to involve the killing of S-phase cells, which are relatively radioresistant (Byfield, 2018) (Ojima *et al.*, 2006). However even non-cytotoxic doses can still increase radiosensitivity, pointing to further mechanisms and it also requires incubation with cells before and during RT. This phenomenon has led to the idea of 5-FU being delivered continuously as an infusion throughout RT treatment regimens (Byfield *et al.*, 1982) (T. *et al.*, 1989).

The data from several 5-FU based phase II–III trials that included more than 3000 patients showed that the pCR rate was approximately 13%. Interestingly, the statistically significant

factors conferring a higher rate were additional chemotherapy agents and 5-FU delivered via an infusion (Hartley *et al.*, 2005)

Capecitabine

There was a clinical need for an oral 5-FU to be developed to overcome the healthcare cost implications with intravenous administration. Capecitabine (Xeloda®; Roche, Basle, Switzerland) is an oral prodrug of 5-FU that undergoes an enzymatic conversion into an active form. The final step in the metabolism is mediated by the enzyme thymidine phosphorylase, which is preferentially upregulated in cancer tissue and as it readily passes through the intestinal mucosa it can therefore have more selective effects within tumour (Ishikawa *et al.*, 1998).

Phase III trial data of 400 patients with LARC of capecitabine vs 5-FU demonstrated that the rate of distant metastasis was 9% lower in the capecitabine group and an increased 3- year DFS with comparable overall 5-year survival and local recurrence rates (Hofheinz *et al.*, 2012).

1.10.3 Additional chemotherapy agents

There are several other chemotherapy agents that can augment the effects of RT on cancer that are not 5-FU based. These include oxaliplatin, irinotecan and poly (ADP- ribose) polymerase (PARP) inhibitors.

Oxaliplatin

Oxaliplatin is a third-generation platinum-based drug that enhances radiation-induced cytotoxicity via irreparable DNA damage through various mechanisms, including formation of inter-strand and intra-strand crosslinks (Martin and Bekaii-Saab, 2013). Pre-clinical data has also determined a synergistic effect of RT with oxaliplatin (Hermann, Rave-Fränk and Pradier, 2008) which has formed the basis of subsequent trials. Several large Phase III trials have

investigated the potential benefits of fluoropyrimidine based RT with or without additional oxaliplatin, however only the CAO/ARO/AIO-04 trial (Sauer *et al.*, 2012)showed any improvement in pCR with additional oxaliplatin therapy. However, the current evidence does not support oxaliplatin use routinely as the benefits are inconsistent and there are associated increased drug toxicity effects (Hill *et al.*, 2012).

Irinotecan

Irinotecan, a topoisomerase (TOPO) 1 inhibitor, inhibits DNA replication through interfering with breaking and re-ligation of DNA strands. Similarly to oxaliplatin, preclinical data demonstrated potent anti-tumour effects as a RT sensitizer (Bosciai *et al.*, 1993).

Trial data is limited to smaller Phase I and II trials where there is evidence from two groups that there is an increased overall survival in patients with LARC through additional irinotecan therapy (Mohiuddin *et al.*, 2013) (Gollins *et al.*, 2011). The ongoing Phase III UK ARISTOTLE trial will aim to answer the question of a potential benefit of irinotecan in addition to capecitabine in LARC.

Poly(ADP-ribose) polymerase (PARP) inhibition

PARPs play a critical role in the recognition and repair of DNA single- and double-strand breaks. Some cancer cells have upregulated PARP activity (Ossovskaya *et al.*, 2010) which has made this enzyme a target to inhibit tumour cells ability to recognise and repair DNA strand breaks generated by RT. Preclinical data has shown that CRC cell lines are radio-sensitized with PARP inhibition, however this may be dependent on BRCA1/2 status (mutant) and on the efficiency of HR. (Page and Yang, 2010) (Verhagen *et al.*, 2015).

Veliparib (ABT-888), a potent orally bioavailable PARP-1/2 inhibitor, has been shown to enhance the antitumor activity of chemotherapy and RT on CRC models (Shelton *et al.*, 2013). Veliparib, in both *in vivo* and *in vitro* studies, had radiosensitizing effects in CRC cells

particularly when combined with irinotecan. As such, PARP inhibitors could play an important role in CRC treatment.

1.10.4 Altered mode of delivery to improve radiotherapy response

"Dose Escalation" is where additional boosts of radiotherapy are delivered in addition to standard treatment regimes. There is evidence to support a direct relationship between pCR and increased radiation dosing (Appelt *et al.*, 2013). However randomized trial evidence is still lacking and there are two trials, the UK "A Phase II trial of Higher RadiOtherapy Dose In The Eradication of early rectal cancer" APHRODITE and "organ preservation in rectal adenocarcinoma" OPRA trials, that will aim to address if there is any superiority in dose escalation therapy in LARC. Other future options may be to alter whether or not traditional photon beam therapy may be replaced by more novel proton beam therapy. This is due to the property that protons can deliver their maximal ionizing therapy at more precise distances than photons with minimal damage to surrounding tissue, this phenomenon is known as the "Bragg Peak"(Vitti and Parsons, 2019). Proton beam therapy has been proved extremely effective in treatment of ophthalmic malignancy as a result (Kacperek, 2009).

There is also increasing evidence to support a role for "total neoadjuvant therapy" in LARC, meaning pre-operative chemotherapy plus CRT for specific cases, however there is still insufficient level II or III trial data to support it as conventional treatment (Franke *et al.*, 2018). Furthermore, we know that local recurrence rate with current treatment is around 5% but distant metastatic rates are up to 30%. Therefore, the question has been raised of whether or not neoadjuvant chemotherapy alone could provide additional systemic therapy to reduce rates of distant metastatic spread without the significant morbidity associated with radiotherapy. Some studies have shown that DFS is improved with neoadjuvant chemotherapy *vs* standard CRT with comparable downstaging effects (Gollins *et al.*, 2018b).

1.11 Assessment and predicting the response to radiotherapy

1.11.1 Assessment of response

Neoadjuvant CRT (NCRT) is considered the standard of care for LARC. It is well recognized that the response to neoadjuvant CRT is both variable and unpredictable for the individual patient, and techniques to risk-stratify patients and predict response are the main focus of this study. Favourable responses to CRT are independently associated with conferring a long-term survival advantage to patients who undergo resection, and in more recent years the possibility of deferral of surgery and organ preservation has also been raised (Renehan *et al.*, 2016).

A complete response to CRT may be classified as either a clinical complete response (cCR) or a pathological complete response (pCR). Although the two terms are often used interchangeably, these responses are assessed differently, and one does not necessarily imply the other. A pCR is based on pathological findings after resection, commonly using the Dworak or Mandard tumour regression grading systems (TRG). A cCR is defined according to a combination of clinical examination (including digital rectal examination), radiological (in particular diffusion-weighted MRI) and endoscopic appearances.

MRI is the best modality for detailed imaging of rectal cancers and to assess downstaging clinically, however downstaging can also occur when tissue is resected and viewed histologically to assess for tumour response (pathological response). LCRT has been shown to have increased downstaging effects but timing is important. The Lyon R90-01 trial ((Cotte *et al.*, 2016) showed that delaying surgery to 6-8 weeks post therapy increased downstaging compared to shorter periods. SCRT may also be combined with a delayed interval to surgery. The recent Stockholm III trial demonstrated improved tumour regression over traditional shortcourse treatment (Erlandsson *et al.*, 2019) through a delay of for 4-8 weeks post SCRT was optimal for downstaging.

A recent short study by the UK National Bowel Cancer Audit (2017) revealed that the median time from completion of CRT to surgical resection is currently 11 weeks in the UK, with the suggestion that the interval between CRT and surgery is becoming more established (Clifford *et al.*, 2018).

There is a growing body of evidence from the initial work by Habr-Gama (Habr-Gama *et al.*, 2004) to support the role of NCRT as the sole treatment for rectal cancer with some patients undergoing an apparent cCR. This has peaked further interest in the role of organ preservation in rectal cancer (Dossa *et al.*, 2017). For those cohorts of patients with an apparent cCR, they may be suitable for a "watch and wait" surveillance with the caveat of recurrent disease that may then require salvage surgery (Renehan *et al.*, 2016).

1.11.2 Predicting a response in rectal cancer

Approximately 10-20% of patients undergoing NCRT will also experience a pCR (Ryan *et al.*, 2015) on histological examination of resection specimens. This data highlights the importance for biomarkers in predicting how patients might respond to NCRT (Tiernan *et al.*, 2014) and the need for improving NCRT to improve outcomes and benefit that this therapy might offer balanced against the associated morbidity risks (Bosset *et al.*, 2004).

At present, there are no predictive molecular biomarkers for CRT response in rectal cancer in clinical use. The most reliable predictor of an increased response is tumour stage, with early tumours more likely to display a cCR. The use of CRT in combination with local excision is perhaps becoming better defined in early T1 rectal cancers, but its value in more advanced cancer is less clear (Borstlap *et al.*, 2018). Trials, such as the STAR-TReC trial (ISRCTN14240288), will compare three different strategies for more advanced tumours up to T3b N0, and assess the feasibility of randomizing to a trial with organ preservation arms. However, the role of neoadjuvant CRT as sole treatment for even more locally advanced tumours that perhaps threaten the circumferential resection margin (CRM) is unknown and further evidence is needed.

1.11.3 Molecular Biomarkers in tumour tissues

DNA mutations and DNA methylation

The two main genes that have been studied in CRC are TP53 and KRAS. A meta-analysis of 30 studies with almost 2000 CRC patients has shown that wild type TP53 was associated with favourable NCRT response (Chen *et al.*, 2012). However, other studies have shown that there is a higher prevalence of TP53 mutations in patients with poorer response to therapy, with NCRT acting as a selection pressure for aberrant p53 protein expression (Sakai *et al.*, 2014).

There is also evidence that specific KRAS mutations in particular codon 13 can cause different effects on rectal cancer resistance to CRT, revealing a complex interplay between KRAS and TP53 (Duldulao *et al.*, 2013).

As described earlier, a specific distinct molecular subtype of CRC is characterized by DNA hypermethylation in CpG-rich promoters (CpG island methylator phenotype; CIMP). There is evidence both *in vivo*, *ex vivo* and *in vitro* that suggests that both hyper- and hypomethylation may predict outcomes and response to treatment and that demethylation of tissues may improve response to IR (Williamson *et al.*, 2015).

Gene Expression

There have been several gene expression profiling studies completed using tissue from patient rectal cancer tissue to investigate any particular genetic signatures associated with CRT response. (Watanabe *et al.*, 2010). and (Agostini, Janssen, *et al.*, 2015) identified multiple different sets of gene signatures that varied between responders and non-responders using pretreatment tumour biopsy samples. X-ray repair cross-complementing protein 3 (XRCC3) is a gene involved in DSB repair through the HR pathway along with Rad51. It has been identified as particular gene of promise as it was identified in tissue from non-responders, and

subsequent small interfering RNA (siRNA) knockdown of this gene improved chemosensitivity to 5FU in colon cancer cells (Agostini, Zangrando, *et al.*, 2015).

Metabolites and proteins

Tumour protein expression has been investigated extensively to identify potential predictive biomarkers. Epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), p21, BCL2-associated X protein (Bax), B-cell CLL/lymphoma 2 (Bcl2), marker of proliferation Ki-67 (ki-67), p53, cyclooxygenase-2 (COX-2), hypoxia-inducible factor 1- α (HIF1- α), thymidylate synthase, E-cadherin, matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2 have been demonstrated to be involved with response to nCRT (Ryan *et al.*, 2016)(Kim and Hur, 2015)(Molinari *et al.*, 2015). Further evidence suggests that these proteins are involved in pathways dysregulated by CRT such as DNA repair, cell cycle progression, cell proliferation and apoptosis.

Tumour microenvironment

The host immune system is known to play an important role in CRT and its role with regards to nCRT has been investigated as a predictor of response. Teng et al. investigated the subset densities of tumour-infiltrating lymphocytes (TILs), as well as programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte protein 4 (CTLA4) expression, before and after nCRT in 62 rectal cancer patients. Patients with high CD8+ TILs, high CD4+ TILs, and low MyeloidDerived Suppressor Cells (MDSCs) achieved good response to nCRT (Teng *et al.*, 2015). McCoy et al. evaluated the subset densities of TILs in post-nCRT surgical samples from 128 rectal cancer patients, and found that Foxp3+ cell density was significantly associated with pCR and improved survival (McCoy *et al.*, 2015).

microRNA

MicroRNAs (miRNAs) are a class of 18-27-nucleotide single-stranded RNA molecules that negatively regulate the expression of specific target genes at the post-transcriptional level. Deregulation of microRNAs (miRs) in CRC has been associated with tumour diagnosis, prognosis, and response to therapies, indicating that they might be promising biomarkers in clinical application (Kong *et al.*, 2012). Gaedcke et al. examined miRNA profiles of tumour biopsies and normal mucosa from 57 rectal cancer patients before treatment, and demonstrated 49 differentially expressed miRNA between normal and cancerous tissues. In addition, expression levels of miR-135b were significantly correlated with tumour regression grade and disease-free survival (Gaedcke *et al.*, 2014). Similarly, miRNA 21 has been shown to induce CRT resistance through altering 5-FU metabolism (Valeri *et al.*, 2010) and tumour analysis of 92 patients with LARC showed that miR-21 is deregulated in rectal cancer patients and that its preoperative expression levels predict pathological response (Caramés *et al.*, 2015).

1.11.4 Molecular biomarkers in blood

Carcinoembryonic antigen (CEA)

CEA is a broadly recognized biomarker for prognosis and monitoring in CRC, however there is evidence that it can be used as a serum predictive biomarker of nCRT response. Zeng et al. found that in 300 patients undergoing curative surgery following nCRT for LARC, that the CEA level was significantly higher in the non-pCR group than in the pCR group. Specifically, 76.0% of the patients with a pCR had a normal pre-treatment CEA level, versus 58.5% of the patients in the non-pCR group (Zeng *et al.*, 2015). In addition, a normal pre-treatment CEA level was significantly associated with pCR in both univariate and multivariate analyses and this has been corroborated in several other studies (Garland *et al.*, 2014) (Kim *et al.*, 2015) (Song *et al.*, 2016).

Raised carbohydrate antigen 19-9 (CA19-9), whilst mostly used in the prognosis and monitoring of pancreatic cancer, has also been shown to correlate with poorer overall survival in a study of 300 rectal cancer patients (Zhang *et al.*, 2015).

Fibrinogen is a glycopeptide synthesised in hepatocytes that plays a crucial role in the clotting cascade as it is converted into fibrin through the activation of thrombin. In a study of almost 1000 patients it was demonstrated that high pre-treatment fibrinogen levels correlated with poorer responses to CRT, although the mechanism remains unknown (Lee *et al.*, 2015). microRNA(miRNA), circulating tumour cells (CTCs) and circulating free nucleic acids miRNAs can also be detected in serum, and heterogeneity has been demonstrated in miRNAs in serum expression between normal cells and cancer cells mirroring data from tissue miRNA expression. In particular two miRNAs, miR-125b and miR-345, have been identified in studies that are associated with poorer nCRT responses in CRC (Yu *et al.*, 2016) (Alix-Panabières and Pantel, 2014).

CTCs have been detected in serum from patients with rectal cancer and not in healthy controls. The levels of CTCs have also been shown to vary on patients with poor or favourable responses to nCRT, with interesting data showing that responders had a higher baseline CTC level than non-responders. However, responders then displayed a more significant drop in CTC levels post nCRT compared to non-responders (Sun *et al.*, 2013). Similar results from the same group also showed that circulating free DNA (cfDNA) levels were significantly higher in rectal cancer patients compared to healthy controls, and that methylation of the cfDNA detected was higher in responders compared to non-responders (Sun *et al.*, 2014).

Immune signalling molecules

Cytokines, including interleukin (IL)-6 and IL-8, have been associated with nCRT responses in rectal cancer . High IL-6 levels are associated with more advanced disease and poorer survival (Lim *et al.*, 2015). Tada *et al*. showed that cytokine levels pre -treatment did not show

any significant variation however post-treatment, levels of IL-6 and tumour necrosis factor (TNF) after nCRT were significantly higher in non-responders compared to responders (Tada *et al.*, 2014).

Single Nucleotide Polymorphisms (SNPs)

Whole genome studies have identified that nine SNPs are responsive to nCRT, the two most important are *CORO2A rs1985859* and the putative marker *FAM101A rs7955740* which may have predictive potential (Kim *et al.*, 2013). In over 100 rectal cancer patients, Nikas *et al.* showed that the homozygous C/C genotype in 5,10-methylenetetrahydrofolate reductase

MTHFR gene (involved in folate metabolism) were 3 times more likely to respond to and 3.25 times more likely not to have recurrence than either the heterozygous or other homozygous genotypes (Nikas *et al.*, 2015). Nelson *et al.* investigated SNP located in the promoter region of the thymidylate synthetase (TS) gene, which potentially metabolism of 5-FU, and found that patients with TS polymorphisms were more likely to have a complete or partial pathologic response to nCRT containing 5-FU (Nelson *et al.*, 2016).

As discussed previously, miRNA expression has been demonstrated as a potential method of predicting response and SNPs in genes targeted by miRNA can have effects on nCRT response, particularly mismatch repair genes (*MLH3*, *MSH3*, *MSH4*, *MSH6*, *PMS1*, *PMS2* and *PMS2L3*) and genes coding for mucin expression (Vymetalkova *et al.*, 2017).

Tissue Markers	Blood Markers
DNA mutations and DNA methylation - TP53 wild type associated with favourable NCRT response (Chen at al 2012) - KRAS mutations alter effect on CRT resistance (Duldulao et al 2013) - CpG island methylator phenotype (CIMP) status displays variable CRT response (Williamson et al 2015)	**CEA** - CEA level was significantly higher in the non-pCR group than in the pCR group post nCRT (Zeng et al 2015) - Normal pre-treatment CEA level was significantly associated with pCR (Garland *et al.*, 2014) (Kim *et al.*, 2015) (Song *et al.*, 2016). **CA 19-9** - Associated with poorer overall survival in 300 rectal cancer patients (Zhang et al 2015) **Fibrinogen** - High pre-treatment fibrinogen levels correlated with poorer responses to CRT, although the mechanism remains unknown (Lee *et al.*, 2015).
Gene Expression - XRCC3 expression associated with poorer CRT response (Agostini et al 2015)	**miRNAS** - miR-125b and miR-345, associated with poorer nCRT responses in CRC (Yu *et al.*, 2016) (Alix-Panabières and Pantel, 2014)
Metabolites and proteins - EGFR, VEGF, p21, Bax, Bcl2ki-67, p53, COX-2, HIF1- α, MMP-9, MMP-2 all associated with response to nCRT through DNA repair and cell cycle pathways (Ryan et al 2016) (Kim and Hur 2015) (Molinari et al 2015)	**CTCs and cfDNA** - Higher drop in CTC levels pre and post nCRT associated with favourable response (Sun et al 2013). - (cfDNA) levels were significantly higher in rectal cancer patients compared to healthy controls, and that methylation of the cfDNA detected was higher in responders compared to non-responders (Sun *et al.*, 2014).
Tumour microenvironment - High CD8+ TILs, high CD4+ TILs, and low Myeloid-Derived Suppressor Cells (MDSCs) achieved good response to nCRT (Teng *et al.*, 2015).	**Cytokines** - (IL)-6 and IL-8, have been associated with nCRT responses in rectal cancer, high IL-6 is associated with decreased survival (Lim *et al.*, 2015) - Higher levels of IL-6 and TNF- after nCRT were significantly higher in non-responders compared to responders (Tada *et al.*, 2014).

microRNA	SNPs
- microRNAs (miRNAs) deregulation CRC has been associated with tumour diagnosis, prognosis, and response to therapies (Kong *et al.*, 2012)	- SNP located in the promoter region of the thymidylate synthetase (TS) gene, more likely to have a complete or partial pathologic response to nCRT containing 5-FU (Nelson *et al.*, 2016). - *CORO2A rs1985859* and the putative marker *FAM101A rs7955740* are SNPs of interest with potential predictors of response (Kim et al 2015)
- miRNA 21 has been shown to induce CRT resistance through altering 5-FU metabolism (Valeri *et al.*, 2010)	

Table 1.3 – *Tissue and serum biomarkers of rectal cancer with supporting evidence.*

1.12 Previous Work

Our research group has an established interest in identifying predictive biomarkers of radiotherapy response in rectal cancer. Novel proteomic profiling of 8 rectal cancer patients at the Countess of Chester Hospital (Chester, UK) has previously been undertaken to identify suitable candidate proteins. Patients were selected for as having LARC at multidisciplinary meetings and decision of whether or not they would be suitable for nCRT. Tissue biopsies were taken using flexible sigmoidoscopy pre and post-CRT (1 week) and at the time of surgical resection. Tissue samples were frozen and stored for mass spectrometry analysis to determine differentially expressed proteins between responders and non-responders to CRT (Figure 4). The response was defined by standard tumour regression grading (TRG) as reported by a Consultant Histopathologist. This generated several candidate proteins that were differentially expressed according to TRG response with the aim of trying to ascertain potential predictive biomarkers for CRT response. These proteins were subsequently independently validated using a specifically constructed tissue microarray (TMA) of 111 rectal cancer specimens from 20072015 to show differential protein expression using immunohistochemistry within the tumour

tissue.

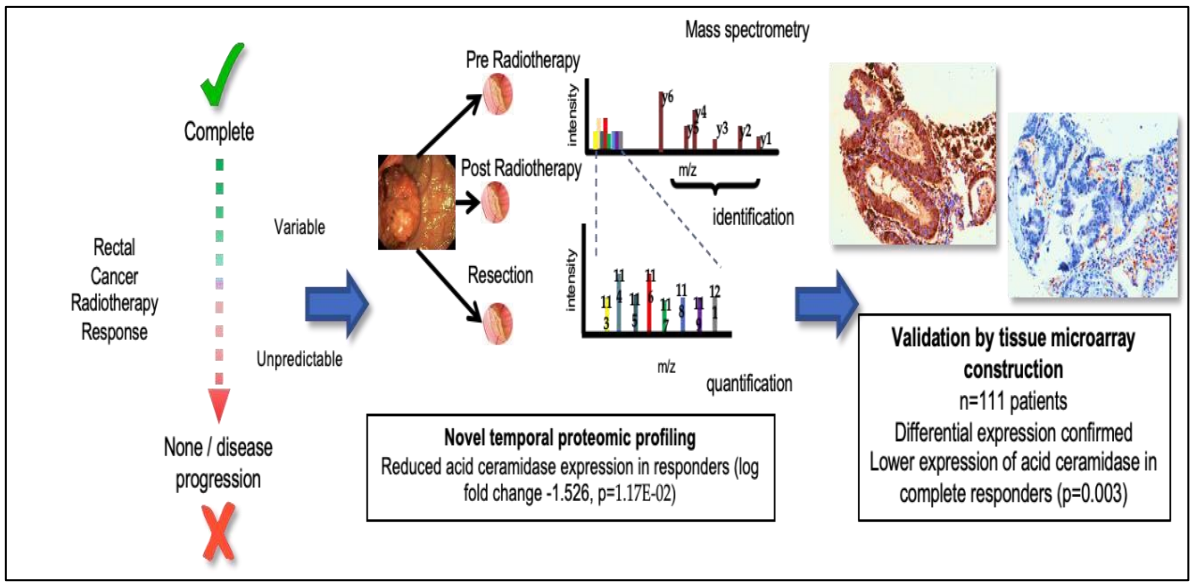


Figure 1.7 – *Previous work on acid ceramidase.* Diagram to illustrate initial proteomic work using tissue samples to identify low AC expression in patients with favourable CRT response then validation using TMA data. (Taken from Bowden, 2018).

Differentially expressed proteins.

Accession Number	Name	*p* value	Log Fold-Change
Q9NZM1	Myoferlin	4.35E−02	−1.633
Q13510	Acid ceramidase	1.17E−02	−1.526
P09525	Annexin A4	1.93E−02	−1.524
P41219	Peripherin	2.13E−02	1.583
P12109	Collagen alpha-1(VI) chain	3.61E−02	1.800
P80748	Ig lambda chain V-III region LOI	4.82E−02	1.866
P07602	Proactivator polypeptide	5.90E−04	1.943
P01860	Ig gamma-3 chain C region	2.74E−02	2.549

Table 1.4 - Table of differentially expressed proteins in rectal cancer using mass spectrometry as identified from previous work.(Taken from Bowden *et al.*, 2018).

As demonstrated, there were three proteins identified that were associated with poorer CRT responses; Myoferlin, Acid Ceramidase and Annexin A4. Myoferlin is a protein that is

expressed in muscle tissue and has been shown to play a role in oropharyngeal cancer (Kumar *et al.*, 2016). Acid ceramidase was selected for further study specifically for two reasons. Firstly it has an established role in several other malignancies (see below) and secondly because there is a commercially available drug acting as an inhibitor; "Carmofur". The latter would allow for manipulation of AC levels within cells to examine if this has any implication on their radiosensitivity.

The major premise of this initial work was to identify potential protein biomarkers in rectal cancer that could predict CRT response. The findings that AC appears to play a role in mediating radiosensitivity in rectal cancer has formed the starting premise for this thesis.

1.13 Acid Ceramidase (AC)

1.13.1 Background to sphingolipids and acid ceramidase

Sphingolipids are key components of cell membranes that maintain structure and function, as well as playing an important role in cell proliferation and cancer (Ponnusamy *et al.*, 2010) (Hannun and Obeid, 2008). There is increasing evidence that sphingolipid metabolism may play a crucial role in targeted anti-cancer therapies (Shaw *et al.*, 2018).

Ceramide is the central molecule of sphingolipid metabolism. Composed of a sphingosine base and amide-linked acyl chains, ceramide serves as the structural and metabolic precursor of more complex sphingolipids, such as sphingomyelin and ceramide-1-phosphate (Figure 6). Ceramide synthesis and metabolism occur in the endoplasmic reticulum and Golgi apparatus, and thus transport of ceramide are of critical importance in the sphingolipid pathway (Perry and Ridgway, 2005). Ceramide itself can be produced either through the hydrolysis of sphingomyelin or synthesised *de novo* in the endoplasmic reticulum.

Ceramide can then be further metabolised by ceramidase enzymes into sphingosine (SPH) and then phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase (SK) (Figure 6).

SPH, ceramide and S1P are generated in response to cellular stresses (chemotherapy, radiotherapy and/or oxidative stress) where they are crucial mediators of cell cycling, death and senescence (Cuvillier *et al.*, 1996) (Lee *et al.*, 1998).

The major enzymes in sphingolipid metabolism have now been fully characterised and altered expression of these enzymes and signalling pathways has been implicated in cancer signalling and regulation (Ogretmen, 2006). There are five different ceramidase enzymes that have been characterized based on their optimum functioning pH level. Congenital acid ceramidase deficiency was identified by Farber in 1952 as a rare metabolic disease of childhood, still less than 100 cases have been reported since its discovery. AC was one of the first to be isolated initially in rats then purified from human urine in 1995 (Bernardo *et al.*, 1995).

1.13.2 Ceramide and apoptosis

Ceramide can accumulate intracellularly in response to stress which can cause cell death. It was first shown in leukaemia cells that accumulation of ceramide caused cell death through apoptosis (Obeid *et al.*, 1993). Ceramide accumulation in mitochondria induces the proapoptotic protein Bax to become recruited, which subsequently activates the caspase pathway and ultimately apoptosis (Chipuk *et al.*, 2012). Ceramide also acts as a second messenger of the apoptotic cascade via apoptosis antigen (CD95) (Grassmé, Schwarz and Gulbins, 2001) and interaction with nitric oxide to cause cell death (Takeda *et al.*, 1999). Ceramide has also been shown to reduce telomerase activity therefore causing telomere shortening, accelerated senescence and apoptosis in lung cancer cells (Ogretmen *et al.*, 2001).

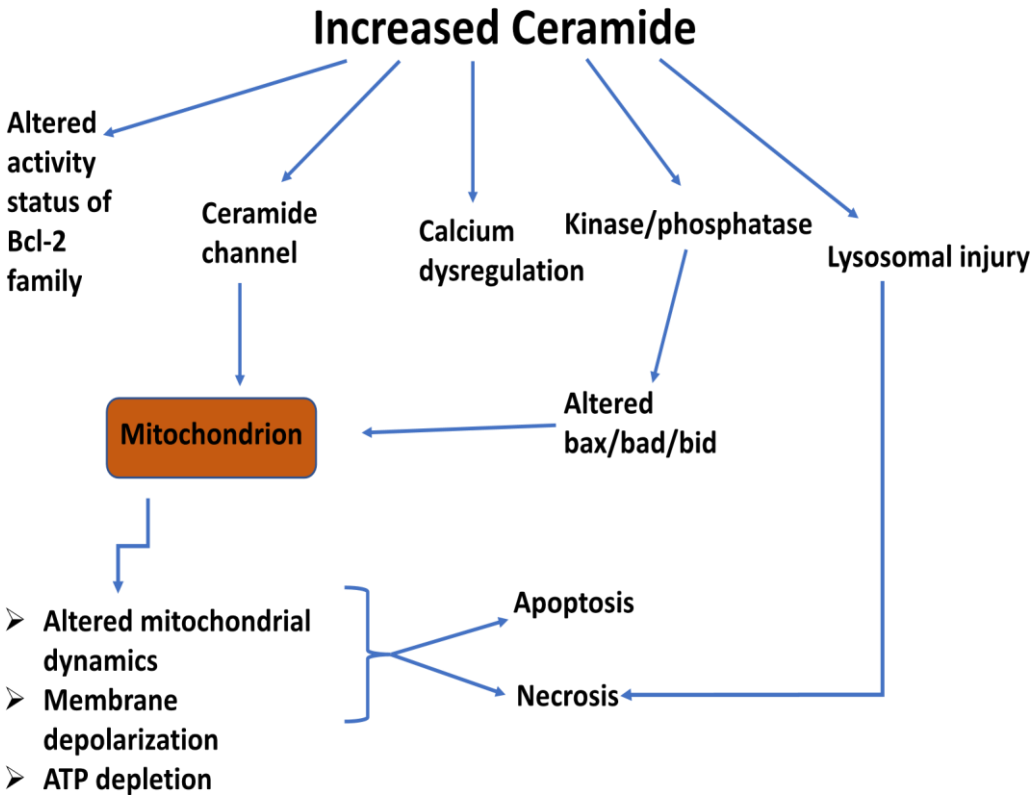


Figure 1.8- ***Diagram to illustrate the role of ceramide in cell death.*** *(Adapted from* Shubhra Chakrabarti *et al.*, 2016)

Many cancer cells have developed methods to extract ceramide from the cell (Truman *et al.*, 2014) in conjunction with upregulation of ceramidase enzymes to metabolise pro-apoptotic ceramide into pro-survival sphingolipids (Patmanathan *et al.*, 2017).

AC itself has been implicated specifically in the development of cancer through two mechanisms:

1. Identification of its over expression in human cancer and/or relationship to stage or prognosis.
2. Observation that its inhibition and consequent rise in ceramide levels leads to apoptotic cell death.

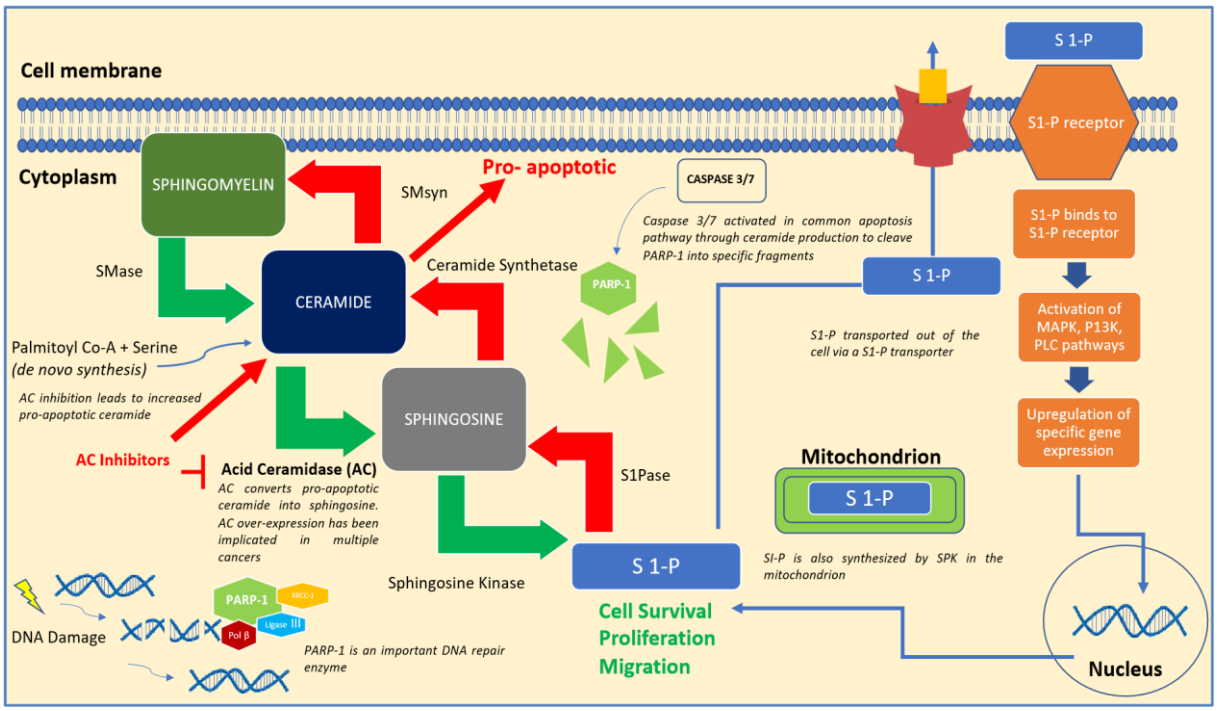


Figure 1.9 - ***"The sphingolipid rheostat".*** *AC converts pro-apoptotic ceramide into sphingosine, which is further metabolised into the pro-survival S1-P sphingolipid. S1-P upregulates various cell proliferation and survival pathways.* (Taken from Govindarajah *et al.*, 2019)

There is a complex interplay between pro-apoptotic ceramide and pro-survival S1P. This is a dynamic system where AC is a crucial enzyme in this pathway that converts ceramide into sphingosine. From this pathway there are several areas that could be manipulated in order to change the balance between cell death and cell survival. This is an ongoing area of research for novel anti-cancer therapy.

1.13.3 AC in cancer

The most established evidence for the role of AC in controlling cell proliferation and radioresistance comes from prostate cancer. siRNA gene knockdown of AC in human PPC-1 cells and targeted drug inhibition with the small molecule inhibitor LCL385 conferred improved radiosensitivity, and conversely upregulation of AC expression conferred increased radioresistance *in vitro* (Mahdy *et al.*, 2009). Increased AC expression has been linked to poorer

tumour response in multiple different cancers including head and neck, myeloid leukaemia, melanoma, hepatobiliary cancers and glioblastoma. In breast and ovarian cancer, AC expression conferred improved outcomes which contradicts evidence from all other cancers. This is thought to be due to a correlation between AC expression and estrogen receptor (ER) expression therefore improved outcome with hormonal therapy (Hanker *et al.*, 2013) (Sänger *et al.*, 2015).

1.13.4 AC in colorectal cancer

The evidence for AC's role in CRC is still not fully elucidated, particularly with only evidence from limited *in vitro* studies. Increased AC expression has been demonstrated in colon cancer cells, and confirmed on immunohistochemical (IHC) analysis compared with normal colonic tissue, and inhibiting AC shown to sensitize cells to oxaliplatin (Klobučar *et al.*, 2018). Other studies have also shown that through inhibiting AC there is increased apoptosis (Baspinar *et al.*, 2017). As described in the previous work from our research group, proteomic and IHC analysis showed that AC expression was associated with poorer CRT response (Bowden *et al.*, 2018).

1.13.5 AC as a therapeutic target in cancer therapy

AC inhibitors have been developed since the 1970s, an important drug in clinical use being Carmofur (1-hexylcarbamoyl-5-fluorouracil). It is a derivative of 5-FU that is an oral prodrug which becomes converted intracellularly to release 5-FU that inhibits thymidylate synthetase and tumour proliferation (Kubota *et al.*, 1991). Carmofur is also a potent AC inhibitor that has been approved for clinical use in Japan since 1981 for adjuvant treatment of colon and breast cancer. However, it is still not approved for use within the UK due to its side-effect profile and there is no trial evidence to support its role in colon cancer. *In vitro* studies of breast and colon cancer have shown promising results (Morimoto and Koh, 2003), however the ability of carmofur to cross the blood-brain barrier is of particular relevance to glioblastoma treatment.

Small molecular inhibitors (SMIs) of AC, such as the LCL family, have shown excellent results *in vitro* in particular melanoma treatment (Realini *et al.*, 2016). However, there are still no reliable and potent AC inhibitors in clinical use available at this time.

For patients with LARC the gold-standard of treatment is for nCRT followed by resectional surgery. Approximately 13% of patients will display a complete pCR with nCRT however there are no clinical biomarkers either in tissue or serum that can risk-stratify patients according to their tumour biology and help predict their response to nCRT. Consequently, there exists a real clinical need to identify potential predictive biomarkers of nCRT response in patients with LARC as a step towards personalised medicine and also to develop new therapeutic targets to improve radiosensitivity.

1.14 Project Hypothesis

Manipulation of acid ceramidase (AC) expression affects the radiosensitivity of rectal cancer cells through an apoptotic-mediated pathway.

1.15 Research Questions

1. Is there differential baseline expression of AC in multiple CRC cell lines?
2. Does baseline AC expression alter the intrinsic radiosensitivity of each respective CRC cell line?
3. Can we manipulate AC expression in CRC cells using biological inhibition (siRNA) and does this translate into differential radiosensitivity phenotypes?
4. If AC expression correlates with CRC cell radioresistance, can we elucidate a potential mechanism?

Chapter 2 Materials and methods

2.1 Cell culture

Initial cell culture techniques of passage, splitting and seeding were all acquired through using HCT-116 p53+ve cells already used routinely in this laboratory.

The following immortalised CRC cell lines: HCT116, HT29, LIM1215, MDST8, GEO and NCI-H716 were kindly donated for this specific work by Professor Ultan McDermott (The Sanger Institute, Cambridge, UK). These cell lines were specifically selected from a screen of 49 cell lines based on their differential N-Acylsphingosine Amidohydrolase 1(ASAH1) mRNA expression (Chapter 2 Materials and Methods Table 2.1). Specific data with regards to intrinsic metastatic potential or invasiveness of each cell line is not fully characterised within the literature however it remains an interesting question especially with regards to any association with baseline AC expression.

HT 29 cells are inherently p53 mutant as a cell line, therefore cannot to produce fully functional p53 through a mutation in codon 273 (He *et al.*, 2015). IR typically induces apoptosis through increased cellular p53 expression leading ultimately to cell death. This detail needed to be considered for this cell line as it may have implications on response of this cell line to radiation (Lee, Blum and Kirsch, 2013).

Cell Line	*asah*-1 (AC) expression
NCI-H716	-2.06
GEO	-1.45

HT-29	-1.25
HCT-116	-0.63
MDST8	1.42
LIM1215	1.55
NCI-H508	1.60

Table 2.1 - *Selected CRC cell lines of variable asah-1 mRNA expression used in this study. Data based from 49 cell lines analysed by the Sanger Institute, Cambridge range from -2.06 (low expression) to +1.6 (high expression), compared with normalised control).*

All cell lines were STR profiled and underwent routine mycoplasma testing. HCT116 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Gillingham, UK) supplemented with 10 % fetal bovine serum, 1 % L-glutamine, 1 % Penicillinstreptomycin and 1 % non-essential amino acids and incubated at 37□C in 5% CO_2. All other cells were cultured in Roswell Park Memorial Institute (RPMI; Sigma-Aldrich, Gillingham, UK) with the same supplements, but additionally with 10 mM hydroxyethyl piperazineethanesulfonic acid (Hepes).

2.1.1 Passaging cells

For cell passage, all cells were grown in T75 flasks and incubated at 37□C in 5% CO_2. Once cells were at approximately ~70–80 % confluence they were suitable for splitting/passaging. The incubation period for all cell lines to achieve this was approximately every 3-4 days and required careful monitoring to ensure that there was no overgrowth.

After the incubation period, media was aspirated, 10ml of cold phosphate buffered saline (PBS) was added then this was aspirated. 1ml of trypsin was added and then the flask ensuring it covered the whole surface, then re-incubated for 5 minutes at 37□C, then 9ml of fresh media was then added again and pipetted to ensure the adherent cells are re-suspended to form a single-celled suspension in 10ml.

For continued growth the cells were passaged at different split ratios (ratio of cell suspension: media) according to their growth rates, faster growing cell lines at a higher ratio and slower growing cells at a lower ratio:

HCT-116 = 1:8

HT-29 = 1: 4

LIM 1215 = 1:4

MDST8 = 1:4

Once the cells were split and placed into a fresh T75 this then changed the "p" or passage number accordingly, cells were not suitable for experiments once they had passed p20.

2.1.2 Cell counting

Cell counting was performed for experiments at the point of passage for various experiments. After the cells had been washed and trypsinised to form a 10ml single – celled suspension. The cell solution was then transferred into a 30ml universal tube and 15µl was removed for counting using a haemocytometer. Three counts were taken each time which gave a cell count of "x" number of cells x 10^4 then this was converted to give a count of "x" number of cells x 10^6 to then calculate the volume of cell suspension to yield 1 million cells for a 10cm dish suitable for seeding and harvesting a cell pellet.

2.1.3 Thawing cells

Cells were all stored in liquid nitrogen for long term use in 1ml cryovials with dimethyl sulfoxide (DMSO). Stored cells were thawed in a water bath at 37°C for approximately 1 minute. Once the liquid had started to thaw, 1ml of media was added to the cells dropwise and gently pipetted to mix. The cell suspension was then transferred to a 15ml falcon tube and 8ml of further fresh media added and pippeted to mix. The cell suspension was then centrifuged at 1500 rpm for 3minutes to create a cell pellet, the supernatant containing the DMSO media. The pellet was then resuspended in 1ml media and transferred to a T75 containing 11ml media. This T75 was usually suitable for splitting and passaging the following day as the cells had reached sufficient confluence.

2.1.4 Freezing cells

One confluent T75 flask would be suitably for two vials of frozen cells for storage. Firstly, media was removed from the flasks, then 8-10ml of PBS was added to wash and then aspirated. 1ml trypsin was added and then flasks incubated at 37°C for 5 minutes. 9ml media was then added and the cell mixture was transferred to a falcon tube, then centrifuged at 1500 rpm for 3 minutes to form a pellet and the supernatant removed. Freezing media was then made with 90%

FBS + 10 % DMSO (1ml needed per vial of cells i.e. 900µl FBS + 100µl DMSO per vial). Cryovials were then labelled accordingly and the pellet was resuspended in freezing medium, ensuring to work quickly to reduce cytotoxic effects of DMSO. 1ml of cell suspension was added to each vial and these were placed into a Mr. Frosty™ (ThermoFisher Scientific, Loughborough, UK) freezing container into a -80°C freezer overnight then transferred into liquid nitrogen for long term storage.

2.2 Cell harvest, protein extraction and quantification

Cell harvest

All Cells were grown in T75 flasks or 10cm dishes (1.2-1.5 million cells/dish) to achieve >70% confluence cells (approximately 4 days growth) and harvested using cell scrapers, washed with 10ml of cold phosphate buffered saline (PBS) transferred into a cooled 15ml tube and centrifuged at 1500 rpm for 5 minutes at 4□C. The cells were then resuspended in 1ml of cold PBS, transferred into a cooled 1.5ml tubes and then centrifuged again at 1500rpm for 5 minutes at 4□C. The supernatant was then aspirated from the cooled 1.5ml tubes to yield the cell pellet, this was then left at -80□C overnight prior to protein extraction.

Protein extraction

Cell pellets were treated using the Tanaka method for protein extraction (Nickson *et al.*, 2017) (Bennett, Madders and Parsons, 2020). 1.5ml tubes were filled with 1ml Buffer I (10 mM Trishydrochloric acid (HCl) (pH 7.8), 200 mM potassium chloride (KCl) and Buffer II (0 mM TrisHCl (pH 7.8), 600 mM KCl, 40 % glycerol, 0.1 mM Ethylenediaminetetraacetic acid (EDTA), 0.2 % Nonidet P-40, 1 µg/ml) respectively then 1µl of 1mM DL-Dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF) and 1µl of of the protease inhibitors (1µg/ml) (aprotinin, pepstatin, leupeptin and chymostatin). To each cell pellet an equivalent packed cell volume (PCV) of Buffer I was added along with twice the volume of Buffer II. Then the cell pellets were rotated in a mini tube rotator for 60 minutes at 4□C before being placed in a cooled centrifuge at 40,000 rpm in for 20 minutes. The whole cell extract (WCE) was aspirated and transferred to cooled 1.5ml tubes for subsequent protein concentration quantification.

Protein quantification

Standard colorimetric Bradford assay was used to confirm the protein concentration. 4ul of cell extract was mixed with 36ul of double distilled water (dd H20) and treated with 960µl of Bradford Reagent (ThermoFisher Scientific, Loughborough, UK) in a cuvette. Controls were made using 960ul of Bradford Reagent with 40ul ddH20 and 40ul of 0.2mg/ml bovine serum albumin (BSA) respectively. The cuvettes were then placed in a 595nm wavelength calibrated spectrophotometer (CECIL Instruments, Cambridge, UK) and each individual cell-line protein concentration was calculated using the formula $0.2/ BSA_{absorbance} \times 10$ = cellular protein mg/ml.

2.3 SDS-PAGE and protein transfer

For the initial baseline AC expression data for all cell lines 40ug of protein was used for immunoblotting analysis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) gels were optimised to 16% for all immunoblotting experiments to ensure the 13kdA

AC fragment was detected. Each gel was a standard 10 – well separating gel at 16% SDSPAGE concentration with a 5% SDS-PAGE stacking gel at the top. Each WCE was added to 10ul of sodium dodecyl sulphate (SDS) loading buffer and water before being heated to 90 °C for five minutes, then loaded onto the 16% SDS-PAGE 10-well gel and run along with 2ul protein ladder marker (BioRad, California, USA) for 110 minutes at 125V using a Mini Gel Tank (Thermo Fisher Scientific, Massachusetts, USA) filled with 1 % Tris-glycine SDS (TGS) running buffer (200ml 10% TGS + 1800ml H_20).

Once the gel had run for the prescribed time and voltage it was then ready to be transferred to a Immobilon FL PVDF membrane (Millipore, Watford, UK). Firstly, the PVDF membrane was activated in methanol for 15s then placed into water for 2 minutes, the water was then removed and replaced with cold transfer buffer (400ml methanol, 200ml 10 X Tris-glycine (TG) made up to 2000ml with water) for > 1 minute. A plate snapper was used to remove the

gel from the cassette and rinsed briefly in cold transfer buffer in a large tub, then the transfer sandwich was set up with sponge, blotting paper and membrane in a Mini Blot Module (Thermo Fisher Scientific, Massachusetts, USA) and left to run for 1.5h at 25V.

Figure 2.1 – ***Protein transfer.*** *Diagram to illustrate protein transfer following electrophoresis prior to immunoblotting.*

2.4 Immunoblotting

The membrane was removed from the module and rinsed in PBS. Then the membrane was blocked for 1h at room temperature with Odyssey blocker (LI-COR Biotechnology, Nebraska, USA) diluted 1:1 with PBS. Membranes were probed with purified primary mouse antibody raised against human ASAH-1 (BD Biosciences: 612302, Wokingham, UK) diluted to 1:1000 concentration and incubated at 4°C overnight. The membrane was then washed with PBS+ 0.1 % Tween-20 then incubated with appropriate secondary anti-mouse antibody Alexa Fluor 680 or IR Dye 800 secondary antibodies (Li-Cor Biotechnology, Cambridge, UK) at 1: 10,000 concentration for 60 minutes at room temperature, washed again with PBS+0.1% Tween-20 and PBS shielded from light then visualised using an Odyssey Image Analysis System (Li-Cor

Biosciences, Cambridge, UK). The membrane was then washed again and re-probed with actin/tubulin antibodies (Sigma-Aldrich, Gillingham, UK) as a loading control at 1:20,000 concentration for 60 minutes at room temperature, washed again and incubated with further appropriate secondary antibody then imaged to allow for semi-quantitative analysis of AC expression using the Odyssey imaging software. All results of AC expression from the current cell lines were normalised to HCT-116 AC expression data as this had been previously validated from previous work.

2.5 Clonogenic assay

The ability of single cells to be seeded onto a plate, incubated, form colonies and then become fixed to enable counting is the basis of the clonogenic assay. This technique was first established in the 1950s (PUCK and MARCUS, 1956). This is now the gold standard method for measuring the radiosensitivity of cancer cells. Baseline clonogenic experiments were performed to establish any association between cellular baseline AC expression and radioresistance. MDST8, HCT-116, HT29 and LIM 1215 cells were used. NCI-H716, NCIH508 and GEO lines were not suitable for clonogenic assays due to their growth conditions. Cells were seeded out into 4 sets of 35mm dishes containing 2ml of complete DMEM or RPMI media including all relevant supplements at 150 000 cells per dish (HCT – 116), 200 000 cells

(MDST8, HT29) or 600 000 cells (LIM 1215). These dishes were then incubated at 37°C for 48h to achieve approximately 50-60% confluence.

Cells were then irradiated as a monolayer at 1,2 and 4Gy respectively using a Faxitron irradiator (Faxitron Bioptics LLC, Arizona, USA) at a dose of 0.05 Gy/minute, along with unirradiated control groups. The media was then aspirated from each irradiated dish, cells were washed with 2 ml PBS. 200ul of 0.25% trypsin added to each dish and incubated for 2 minutes, then 800ul of media added to form a cell solution of 1ml and transferred into cooled 1.5ml tubes. The cell

density was then counted manually using a haemocytometer. Cell suspensions were then diluted with media based on the counts to form a stock cell suspension at each radiation dose and plated at increased densities with radiation dose onto standard Nunc 6 – well clonogenic plates (Thermo Fisher Scientific, Massachusetts, USA) and incubated for variable periods (≈ 8 days HCT-116/MDST8 *vs* ≈ 10 days HT29/LIM 1215).

Seeding densities were adjusted to yield the following final counts and incubation periods used for each cell line for baseline clonogenic assays (Chapter 2: Materials and Methods Table 2.2); numbers denote number of cells seeded in top and bottom row respectively along 6-well plate). The cells were then stained and fixed with crystal violet and the colonies were counted using a GelCount (Oxford Optronix, Oxford, UK) automated plate imager for subsequent data analysis.

Radiation Dose/Gy	**MDST8**	**HCT-116**	**HT 29**	**LIM 1215**
0 (control)	*500* *1000*	*250* *500*	*250* *500*	*250* *500*
1	1000 2000	500 1000	500 1000	500 1000
2	2000 4000	1000 2000	1000 2000	1000 2000
4	4000 8000	2000 4000	2000 4000	2000 4000
Approximate Incubation Period/ Days	**8**	**8**	**10**	**10**

Table 2.2 - *Cell seeding counts for 6-well plates and incubation periods for all cell lines.*

Relative colony formation (surviving fraction) was expressed as colonies per treatment level versus colonies that appeared in the untreated control. Plating efficiencies for the cells were as followed: - HCT-116 (45 %), HT 29 (40 %), LIM 1215 (30 %) and MDST8 (14%). Surviving fraction curves were then produced for each cell line at each particular dose of radiation with statistical analysis performed using "CFAssay for R" software package (Braselmann *et al.*, 2015) (Chapter 2: Materials and Methods 2.9).

2.6 Small interfering RNA (siRNA) inhibition

RNA interference was first discovered in plants (Hamilton and Baulcombe, 1999). Since that time it has been widely used to induce rapid gene silencing in cell lines with significant therapeutic potential in gene therapy. siRNA interferes with the expression of specific genes with complimentary nucleotide sequences, and functions by causing mRNA to be broken down after transcription through the RNA induced silencing complex (RISC) (Agrawal *et al*, 2003). siRNA is negatively charged and can only be delivered into cells by use of a cationic lipid to form a liposome that can overcome the electrostatic charge of the cell membrane (Dalby *et al.*, 2004).

2.6.1 siRNA transfection and AC expression siRNA inhibition (siAC) was used to assess if the *asah1* gene could be targeted to reduce downstream AC expression. From the work previous done by David Bowden on HCT-116 cells (Bowden, 2018) we selected an individual siRNA ASAH-1 (siAC) sequence (D-005228-03, Horizon Discovery Ltd, Cambridge, UK) that had already been shown to downregulate AC expression from a pooled group of oligonucleotides. HCT-116, MDST8, HT29 and LIM1215 cells were seeded onto 35mm dishes in their respective media at either 100 000, or 200 000 cells/dish. The cells were then incubated as a monolayer at 37°C. The cells were then inspected and transfected at 24h. For transfection a stock transfection solution was made using RNAiMAX (Life Technologies, Paisley, UK) and DMEM without additives. The stock solution in order for each 3cm dish to contain 2.5µl RNAiMAX + 125µl DMEM. Each dish also contained siAC (D-005228-03, Horizon Discovery Ltd, Cambridge, UK) at either 40nM (2.5 µl) siAC for the lower AC expressers (HT29/HCT-116) or 80nM (5µl) for the higher expressers (MDST8/LIM 1215). Lipofectamine only control and non-targeting siRNA sequence controls (Eurogentec, Liege, Belgium) / (AllStars Negative

Control siRNA; Qiagen, Manchester, UK) were also used with non-targeting control concentrations at the equivalent concentration to the siAC for respective cell lines. The RNAiMAX/DMEM transfection solution was then added to the siAC and non-targeting control solutions and incubated for 510 minutes at room temperature then 250 µl of the complete transfection solution was added dropwise to each 3cm dish. Cells were then incubated at 37°C and harvested at 48h. WCEs were prepared for each cell line as detailed in section 2.2 for treatment with siAC or non-tar getting control. These WCEs were then run on 16% SDS-PAGE gel electrophoresis as described in section 2.3 and 2.4 with immunoblotting for AC expression and imaging to produce semi-quantative data for effects of siRNA on AC protein expression.

2.7 Clonogenic assays with biological inhibition of ASAH 1 (siAC)

Clonogenic assays were performed using HCT-116, HT 29 and LIM 1215 cell lines in the standard method (Chapter 2: Materials and Methods 2.5) as described previously in section 2.5, the incubation periods were maintained. Cells were seeded, plated and transfected with siAC at either (D-005228-03, Horizon Discovery Ltd, Cambridge, UK) at either 40nM for HCT116/HT 29 or 80nM for LIM 1215/ MDST8 as these concentrations varied according to baseline AC expression along with RNAiMAX lipofectamine (Life Technologies, Paisley, UK) only controls and non-targeting siRNA (AllStars Negative Control siRNA; Qiagen, Manchester, UK) which was used at the same concentration as the siAC.

Below are the final seeding densities at each radiation dose and transfection treatment that were used in the experiments. The seeding densities had to be optimised; higher counts had to be used for the higher radiation doses for each cell line compared to baseline to account for plating efficiencies and the siAC treatment for the LIM 1215 required significantly higher seeding densities and the radiation doses were also scaled down:

Radiation Dose/Gy	RNAiMAX (lipofectamine only control)	Non-targeting siRNA, QIAGEN (40nM)	siAC (40nM)

0 (control)	*250* *500*	*250* *500*	*750* *1500*
1	500 1000	500 1000	1500 3000
2	1000 2000	1000 2000	6000 12000
4	4000 8000	4000 8000	12000 24000

Table 2.3 - *HCT-116 seeding counts and treatments*.

Radiation Dose/Gy	RNAiMAX (lipofectamine only control)	Non-targeting siRNA, QIAGEN (40nM)	siAC (40nM)
0 (control)	*250* *500*	*250* *500*	*250* *500*
1	500 1000	500 1000	500 1000
2	1000 2000	1000 2000	1000 2000
4	4000 8000	4000 8000	4000 8000

Table 2.4 – *HT 29 seeding counts and treatments*.

Radiation Dose/Gy	RNAiMAX (lipofectamine only control)	Non-targeting siRNA, QIAGEN (80nM)	siAC (80nM)
0 (control)	*500* *1000*	*500* *1000*	*2500* *5000*
0.25	1000 2000	1000 2000	5000 10000
0.5	2000 4000	2000 4000	10000 20000
1	4000 8000	4000 8000	20000 40000

Table 2.5*- LIM 1215 seeding counts and treatments.*

Following their respective incubation periods, cells were stained and fixed with crystal violet and the colonies were counted using a GelCount (Oxford Optronix, Oxford, UK) automated plate imager for subsequent data analysis.

As with the baseline experiments, the assays were analysed using "CFAssay for R" software (Chapter 2: Materials and Methods 2.9) to produce surviving fraction curves for each cell line with respective individual treatment at each dose of radiation and repeated in triplicate.

2.8 Poly (ADP-ribose) polymerase-1 (PARP-1) analysis

PARP-1 plays a critical role in the recognition and repair of DNA single- and double-strand breaks. Cancer cells have demonstrated upregulated PARP activity which has made this enzyme a target to inhibit tumour cells ability to recognise and repair DNA strand breaks from RT. However, PARP-1 itself is also cleaved into specific fragments during apoptosis. These fragments can be readily detected on standard immunoblotting; therefore can be used as a biomarker for cells undergoing apoptosis (Chaitanya, Alexander and Babu, 2010). PARP-1 cleavage was therefore investigated to determine a link between biological inhibition of AC through siRNA and increasing radiosensitivity through apoptosis.

HCT-116 and HT 29 cells were seeded and transfected with siRNA ASAH-1 (D-005228-03, Horizon Discovery Ltd, Cambridge, UK) alongside RNAiMAX lipofectamine (Life Technologies, Paisley, UK) and non-targeting siRNA AllStars Negative Control siRNA; Qiagen, Manchester, UK) using the standard method (Chapter 2: Materials and Methods 2.6.1). These cells were irradiated at 0 (control), 4, 8 and12Gy then harvested at 0h (control), 2h, 6h and 24h and protein extraction was performed using the Tanaka method (Chapter 2: Materials and Methods 2.2). WCEs were SDS-PAGE and immunoblotting as described previously (Chapter 2: Materials and Methods 2.4) and incubated with primary mouse monoclonal antibody raised against human PARP-1 ((5A5): sc-56197, Santa Cruz Biotechnology Inc, Heidelberg, Germany) (1:2500 concentration) at 4°C overnight. The membrane was then

washed with PBS+ 0.1% Tween-20 and PBS then incubated with appropriate secondary antimouse antibody Alexa Fluor 680 or IR Dye 800 secondary antibodies (Li-Cor Biotechnology, Cambridge, UK) (1: 10,000 concentration) for 60 minutes at room temperature, washed again with PBS+ 0.1 % Tween-20 and PBS then visualised using an Odyssey Image Analysis System (Li-Cor Biosciences, Cambridge, UK). The membrane was then washed again and reprobed with actin/tubulin antibodies (Sigma-Aldrich, Gillingham, UK) as a loading control (1:20,000 concentration) for 60 minutes at room temperature, washed again and incubated with further appropriate secondary antibody then imaged to allow for semi-quantitative analysis of AC expression using the Odyssey imaging software.

2.9 Statistical Analysis

Clonogenic assay or colony formation assay (CFA) is the gold standard to determine cell reproductive death after treatment with ionizing radiation. The relationship between the radiation doses and the proportion of surviving colonies is usually described by parametric cell survival curves. "CFAssay for R" uses the commonly used linear-quadratic model (LQ model) (Franken *et al.,* 2006). The software also allows for two-way experimental designs to assess cell survival for a particular line compared to treatment with a therapeutic drug (Aichler *et al.,* 2013). The "CFAssay for R" software package was used for statistical analysis for all clonogenic assay data in this study according to methods described in several other studies (Braselmann *et al.,* 2015). Microsoft Excel was used to produce standard error (SE) bars for each point on the surviving fraction curves. The student's t-test was used for the PARP-1 immunoblotting results to test for statistical significance. A P value less than 0.05 was considered statically significant between the control and treatment groups in all experiments.

Chapter 3

Summary of Results Chapter Aims

Chapter 3: AC expression and modulation

- Is there differential baseline expression of AC in multiple CRC cell lines?
- Does baseline AC expression have an effect on intrinsic radiosensitivity of each respective CRC cell line?
- Can we manipulate AC expression in CRC cells using biological inhibition (siRNA)?

Chapter 4: AC expression and radiosensitivity

- Does manipulation of AC expression alter the radiosensitivity of CRC cell lines?

Chapter 5: Mechanistic work

- If AC expression correlates with CRC cell lines radioresistance, can we elucidate a potential mechanism?

Chapter 3

AC expression and modulation

3.1 Background and previous work

Our research group has an established interest in identifying predictive biomarkers of radiotherapy response in rectal cancer. Novel proteomic profiling of 8 rectal cancer patients at the Countess of Chester Hospital (Chester, UK) has previously been undertaken to identify suitable candidate proteins. Tissue biopsies of patients with LARC were obtained pre and postCRT (1 week) and at the time of surgical resection. Mass spectrometry analysis was used to analyse differentially expressed proteins between responders and non-responders to CRT (Figure 4). Standard tumour regression grading (TRG) was utilized to measure the tumour response to therapy. Several candidate proteins were differentially expressed according to TRG response, and these proteins were subsequently independently validated using a specifically constructed tissue microarray (TMA) of 111 rectal cancer specimens from 2007-2015 to show differential protein expression using immunohistochemistry within the tumour tissue.

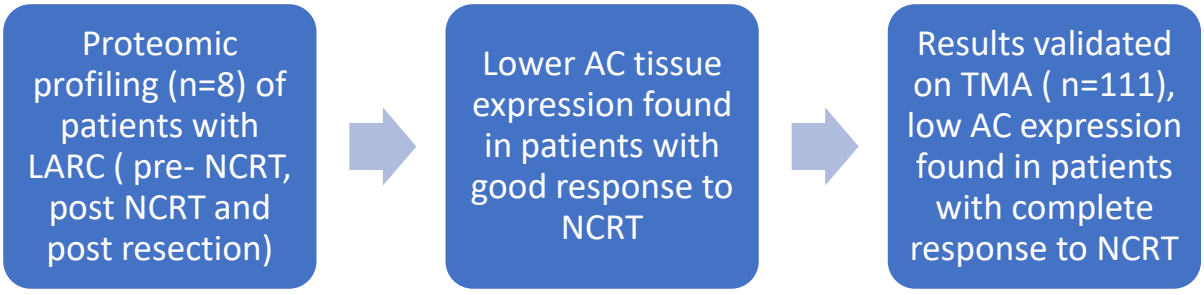


Figure 3.1 – *Previous work on acid ceramidase.* Flow chart to summarise initial proteomic work using tissue samples to identify low AC expression in patients with favourable CRT response then validation using TMA data. (adapted from Bowden, 2018).

Differentially expressed proteins.

Accession Number	Name	*p* value	Log Fold-Change
Q9NZM1	Myoferlin	4.35E−02	−1.633
Q13510	Acid ceramidase	1.17E−02	−1.526
P09525	Annexin A4	1.93E−02	−1.524
P41219	Peripherin	2.13E−02	1.583
P12109	Collagen alpha-1(VI) chain	3.61E−02	1.800
P80748	Ig lambda chain V-III region LOI	4.82E−02	1.866
P07602	Proactivator polypeptide	5.90E−04	1.943
P01860	Ig gamma-3 chain C region	2.74E−02	2.549

Table 3.1 - *Table of differentially expressed proteins in rectal cancer using mass spectrometry as identified from previous work.(Taken from* Bowden *et al.*, 2018).

3.2 AC in cancer

The most established evidence for the role of AC in controlling cell proliferation and radioresistance comes from prostate cancer. siRNA gene knockdown of AC in human PPC-1 cells and targeted drug inhibition with the small molecule inhibitor LCL385 conferred improved radiosensitivity, and conversely upregulation of AC expression conferred increased radioresistance *in vitro* (Mahdy *et al.*, 2009). Increased AC expression has been linked to poorer tumour response in multiple different cancers including head and neck, myeloid leukaemia, melanoma, hepatobiliary cancers and glioblastoma. In breast and ovarian cancer, AC expression conferred improved outcomes which contradicts evidence from all other cancers. This is thought to be due to a correlation between AC expression and estrogen receptor

(ER) expression therefore improved outcome with hormonal therapy (Hanker *et al.*, 2013) (Sänger *et al.*, 2015).

3.2.1 AC in colorectal cancer

The evidence for AC's role in CRC is still not fully elucidated, particularly with only evidence from limited *in vitro* studies. Increased AC expression has been demonstrated in colon cancer cells, and confirmed on IHC compared with normal colonic tissue, and inhibiting AC shown to sensitize cells to oxaliplatin (Klobučar *et al.*, 2018). Other studies have also shown that through inhibiting AC there is increased apoptosis (Baspinar *et al.*, 2017). As described in the previous work from our research group, proteomic and IHC analysis showed that AC expression was associated with poorer CRT response (Bowden *et al.*, 2018).

3.2.2 AC as a therapeutic target in cancer therapy

AC inhibitors have been developed since the 1970s, an important drug in clinical use being Carmofur (1-hexylcarbamoyl-5-fluorouracil). It is a derivative of 5-FU that is an oral prodrug which becomes converted intracellularly to release 5-FU that inhibits thymidylate synthetase and tumour proliferation (Kubota *et al.*, 1991). Carmofur is also a potent AC inhibitor that has been approved for clinical use in Japan since 1981 for adjuvant treatment of colon and breast cancer. However, it is still not approved for use within the UK due to its side-effect profile and there is no trial evidence to support its role in colon cancer. *In vitro* studies of breast and colon cancer have shown promising results (Morimoto and Koh, 2003), however the ability of carmofur to cross the blood-brain barrier is of particular relevance to glioblastoma treatment. Small molecular inhibitors (SMIs) of AC, such as the LCL family, have shown excellent results *in vitro* in particular melanoma treatment (Realini *et al.*, 2016). However, there are still no reliable and potent AC inhibitors in clinical use available at this time.

3.3 Baseline cellular AC expression quantified by immunoblotting

Immunoblotting, specifically Western blotting is a multistep procedure that is one of the most commonly utilized techniques in cell biology for estimating levels of protein expression (He

and Herr, 2010). Cells were grown and harvested, protein was then extracted from each cell line and run on 16% SDS-PAGE gel using electrophoresis. The separated proteins were then transferred onto PVDF membranes and then incubated with primary and secondary antibodies to allow for target protein levels to be visualised and subsequently quantified.

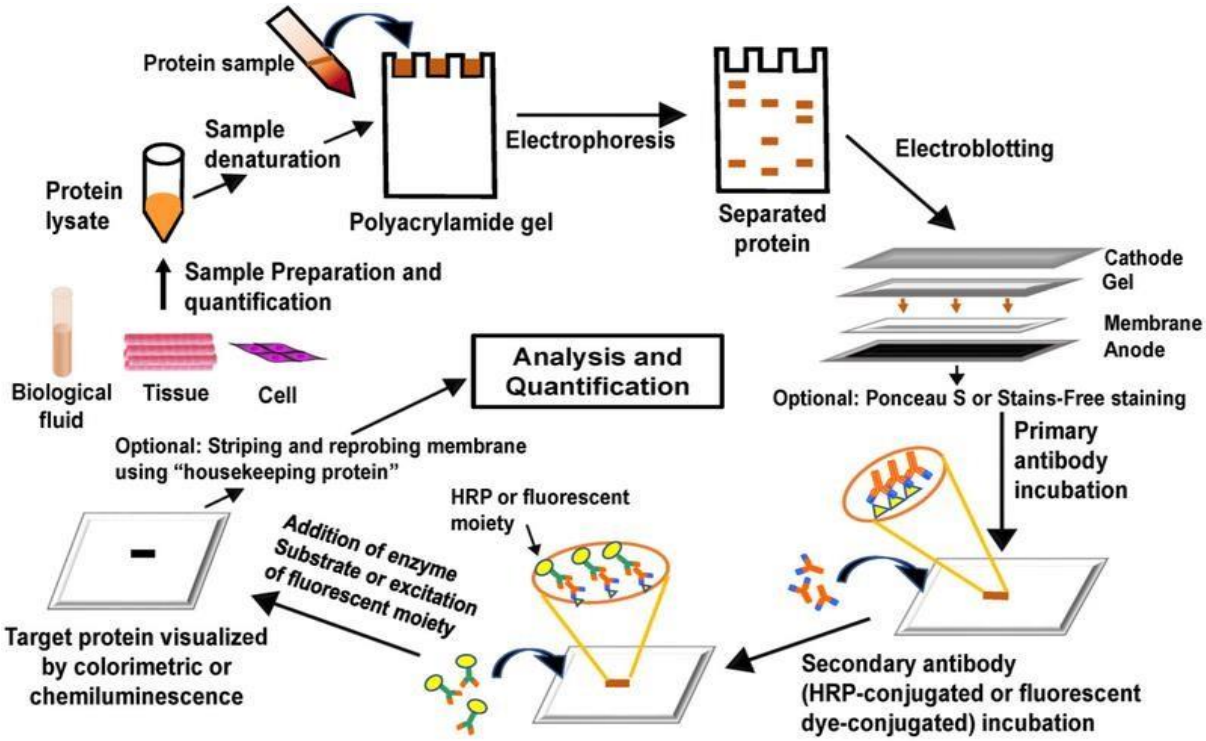
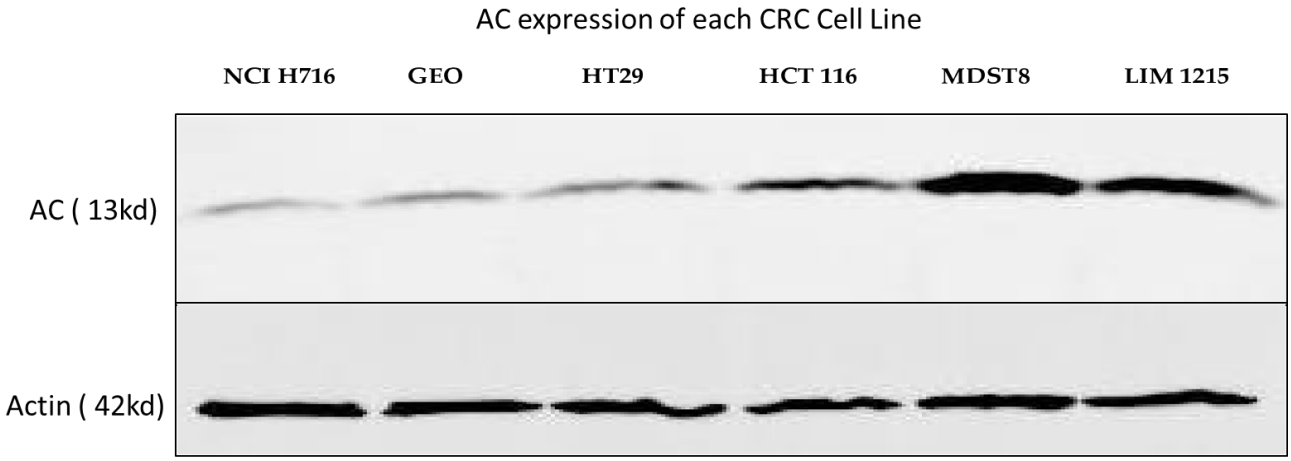


Figure 3.2 – ***Principles of immunoblotting.*** Diagram to illustrate principles of immunoblotting from cell culture and protein harvest to targeted protein visualisation and quantification. (Taken from Mishra, Tiwari and Gomes, 2017)

Experimental question:

Is there differential baseline expression of AC in multiple CRC cell lines?

A



B

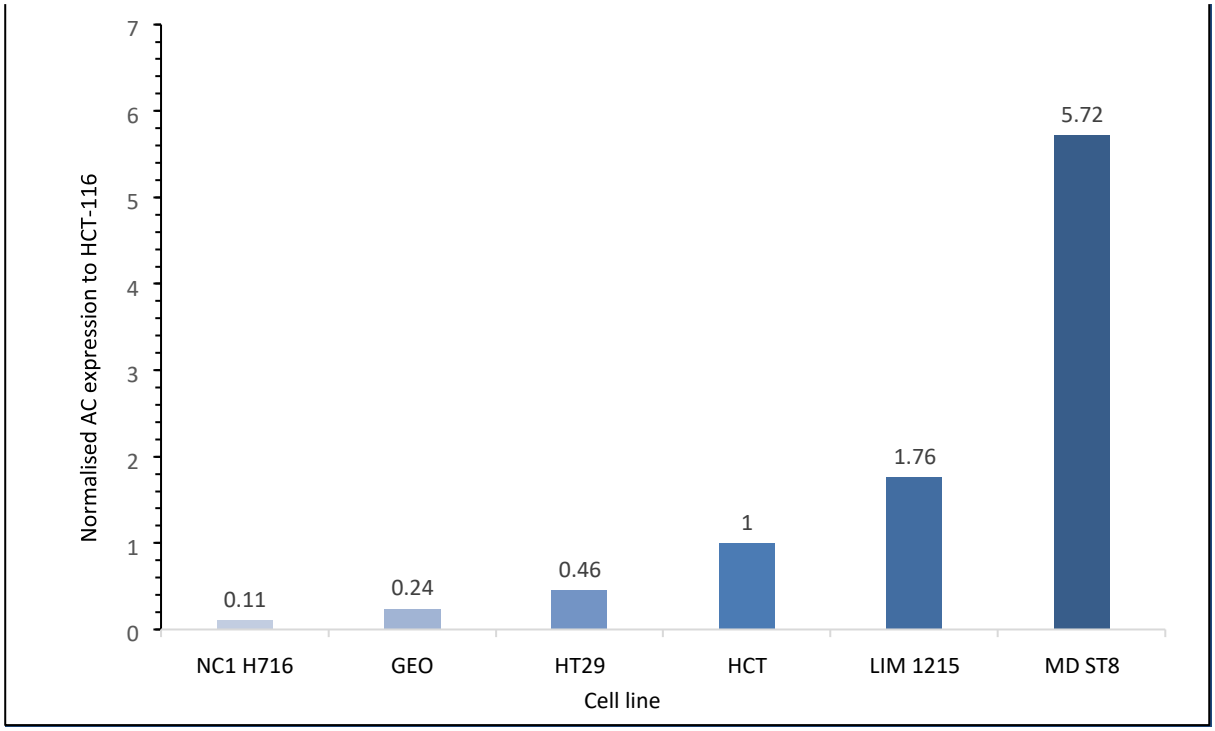


***Figure 3.3* - Differential baseline AC expression across panel of CRC cell lines.** (A) Acid ceramidase is a lysosomal enzyme that is synthesized as a 55kDa precursor protein, which is then further processed into the mature α-subunit (13kDa) and β-subunit (40kDa).

Immunoblotting using 16% SDS-PAGE gel of WCE from six different CRC cell lines shows variable baseline AC expression increasing (L-R). AC fragment seen at 13kD and results normalised to actin expression (42kD). (B) Bar graph to illustrate variable baseline AC

expression across the cell lines (experimental repeats in triplicate) with all values normalised to HCT 116 expression.

Initial baseline immunoblotting across the six cell lines confirmed differential baseline AC protein expression using mouse antibodies raised against human ASAH-1 (BD Biosciences: 612302, Wokingham, UK). AC protein expression was normalised relative to HCT116, and that for example NC1 cells demonstrated ~10-fold less expression whereas MDST8 showed ~5.8-fold higher expression. These results therefore correlate with the data analysed from the initial panel of cells screened by Professor Ultan McDermott with variable mRNA expression for low AC protein expression and LIM 1215 cells displaying high expression. This baseline expression data validated the results provided by the Sanger Institute and formed the basis for all subsequent experiments.

3.3.1 Baseline clonogenic survival assays for CRC cell lines

The clonogenic (or colony forming) assay has been an established technique since the landmark paper published in 1956 using x-ray irradiation for dose response curves in mammalian HeLa cells in culture (PUCK and MARCUS, 1956). The technique allows for differences in reproductive viability of a single cell to form a colony (50 cells or greater) and to compare a control group to another group exposed to exogenous factors (i.e. irradiation, chemotoxic agents) or cells that have been genetically influenced. As a result, this assay has become an important tool in establishing the various survival phenotypes of different cell lines when exposed to different doses of irradiation or chemotherapy (Rafehi *et al.*, 2011). For a standard clonogenic survival assay three parts are involved:

1. Treatment of the cell in a monolayer using tissue culture material
2. Preparation of single cell suspensions and plating an appropriate number of cells on growth plates.

3. Fixing, staining and counting colonies on growth plates following specific incubation periods depending on each cell line (1 – 3 weeks)

Initially baseline clonogenic assays were formed whereby each respective cell line was grown individually on 3cm dishes, irradiated at specific doses then single cell suspensions generated to allow for defined numbers of cells to be plated onto 6 – well plates. Therefore this would allow any difference in intrinsic radiosensitivity to be observed between the respective cell lines.

Experimental question:

Does baseline AC expression have an effect on intrinsic radiosensitivity of each respective CRC cell line?

Radiation Dose/Gy	**MDST8**	**HCT-116**	**HT 29**	**LIM 1215**
0 (control)	*500* *1000*	*250* *500*	*250* *500*	*250* *500*
1	1000 2000	500 1000	500 1000	500 1000
2	2000 4000	1000 2000	1000 2000	1000 2000
4	4000 8000	2000 4000	2000 4000	2000 4000
Approximate Incubation Period/ Days	**8**	**8**	**10**	**10**

Table 3.2 - *Initial Baseline AC experiments seeding counts and incubation periods.*

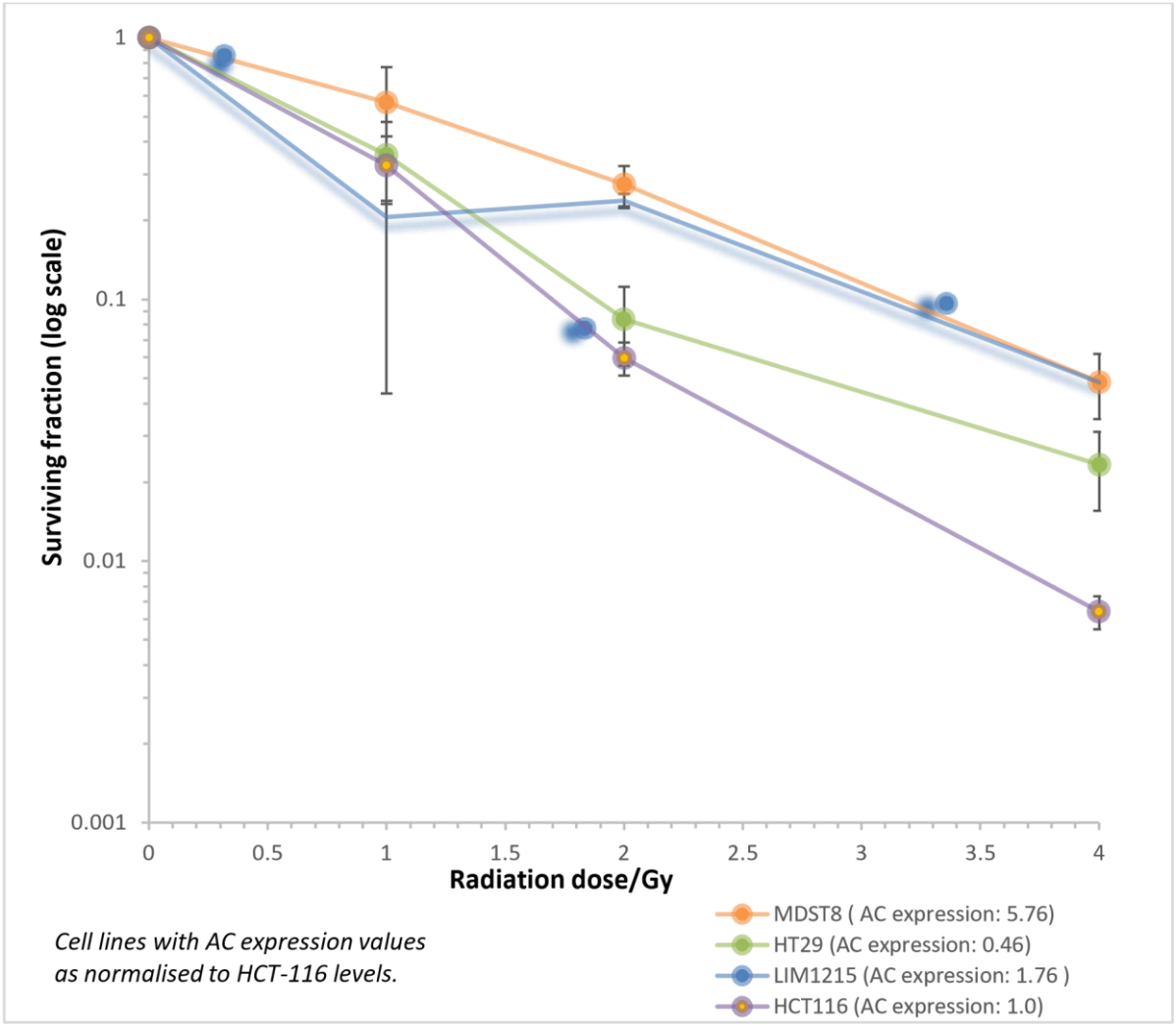


Figure 3.4 - Differential baseline cellular AC expression correlates with radiosensitivity. Cells were irradiated with x-rays and cell survival measured using clonogenic assays. Shown is the mean surviving fraction ± standard error (S.E) on a logarithmic scale from three independent experiments. Cellular AC expression was normalised to HCT – 116 expression. Statistical significance was achieved with survival and baseline AC expression normalised to HCT 116 AC protein levels; HT29 $p < 0.0003$, LIM 1215 $p < 0.000002$ and MDST8 $p < 0.00001$.

Following the initial immunoblotting experiments to validate the ASAH-1 mRNA screening data from The Sanger Institute, cells were cultured, seeded, irradiated and plated at set numbers of colonies as described in the methods. The initial baseline clonogenic survival assays appeared to show that the cell lines displayed variable radiosensitivity at the set doses of x-ray treatment. The HCT-116 cells were generally more radiosensitive than the MDST8 and

LIM1215 cell lines which were comparably more radioresistant. The results have shown that the higher baseline AC expressing cell lines appear to demonstrate a more radioresistant phenotype. Cellular AC expression was normalised to HCT-116 levels as these cells had been studied and validated in the previous work by David Bowden in this group and their ASAH-1 expression was intermediate allowing for variation between high and low expressing cell-lines.

Clonogenic Plate Data for Baseline AC expression

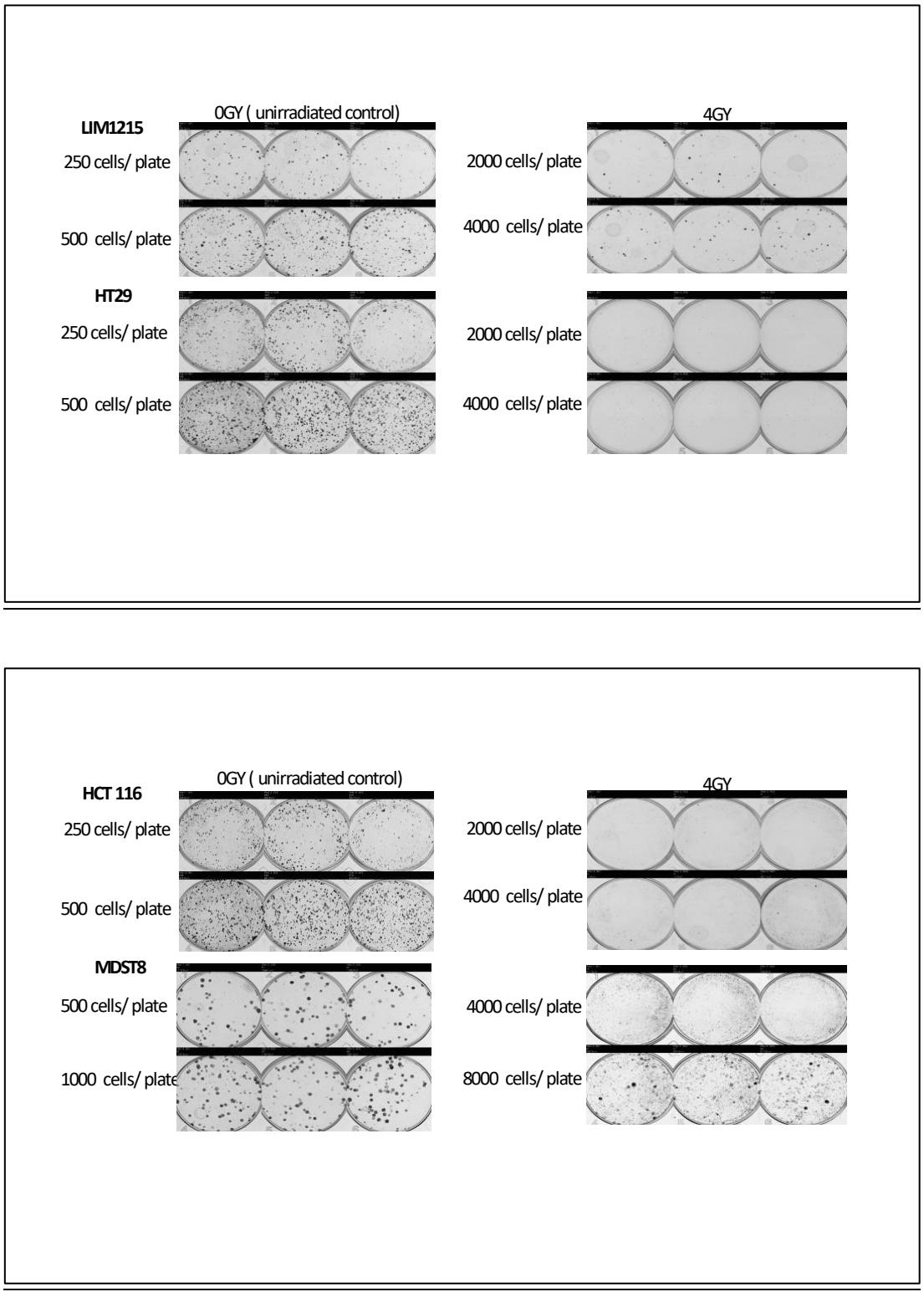


Figure 3.5 - ***Selected clonogenic images from 6- well plates with all four CRC cell lines at 0Gy and 4Gy respectively.*** The images show that the higher AC expressing cell lines (MDST8 and LIM 1215) display higher colony numbers at 4Gy irradiation compared with the lower

expressing cell lines (HCT 116 and HT29). Data from these clonogenic samples was analysed across three repeats to plot the graph as displayed in Figure 3.4.

As cell lines will naturally have varying phenotypic radiosensitivity irrespective of AC expression, these initial experiments would not provide robust evidence to support a role for AC in mediating radiosensitivity. Therefore, AC expression would have to be specifically targeted. In this study, biological inhibition of AC was utilized as detailed previously (Chapter 2 Materials and Methods 2.6.1).

3.4 Biological modulation of AC expression

Small interfering RNAs (siRNAs) have become an important tool in studying gene function within cells. Initially discovered in plant biology as a defence to viral infection it was not until 1998 where the introduction of specific targeted siRNA into the nematode *C.elegans* induced observable functional changes in muscle protein within the organisms (Fire *et al.*, 1998).

siRNAs are typically around 22 nucleotides in length and correspond to a specific mRNA sequence to a parent gene. In nature, double stranded RNAs (dsRNAs) can be made endogenously but these can also be experimentally introduced into a cell. The dsRNAs are cleaved by an RNAse III family of enzymes called “Dicer” (Bernstein *et al.*, 2001) within the cell into single strand RNAs, which then subsequently bind to RNA binding protein called “Argonaute”, this in turn forms an RNA induced silencing complex (RISC) that directly binds to the corresponding mRNA and ultimately induces gene silencing.

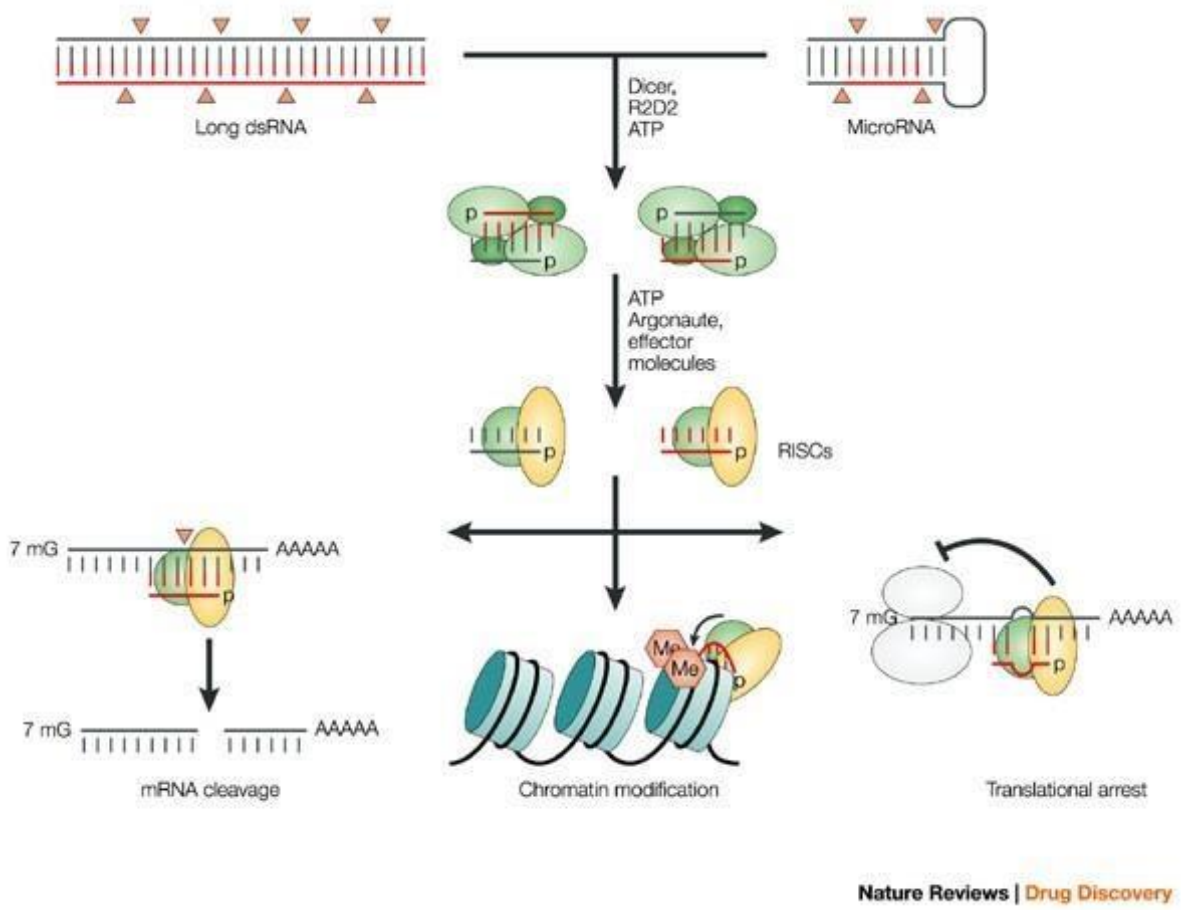


Figure 3.6 - siRNA induces gene silencing. Illustration to show dsRNA or micro RNA is cleaved and processed through Dicer and then bound to Argonaute to form the RISC complex that induces gene silencing through mRNA cleavage, chromatin modification or translational arrest. (Taken from Dorsett and Tuschl, 2004).

Experimentally, the challenge has been to safely package siRNA and ensure it is transported into the cell without damage so it can exert its desired effect. There are two main methods for this, namely viral and non -viral vectors. Liposomes in particular, have proven to be efficient methods of transporting siRNA into cells where it was first employed in 1987 to transport nucleic acids into cells within tissue culture (Felgner *et al.*, 1987). Although siRNA is specific to mRNA sequences there exists "off-target" effects through areas of homologous sequence that has potential to bind to other mRNA sequences and also through immunomodulatory mechanisms (Watts, Deleavey and Damha, 2008).

Experimental Question:

Can we manipulate AC expression in CRC cell lines using biological inhibition (siRNA)?

3.4.1 Targeted inhibition of AC through siRNA

All four CRC cell lines were transfected with siRNA for the ASAH-1 gene (ACsi) and a non-targeting siRNA (NTsi). For the HCT 116 and HT29 cells, 40nM of siRNA was used and 80nM of siRNA was used for the higher AC expressing cell lines (MDST8 and LIM 1215), to ensure proportionate transfection to induce AC gene silencing.

Once all the cell lines had been transfected with ACsi or NTsi they were then left for 48h and then harvested and whole cell extracts were prepared. Standard immunoblotting, specifically Western blotting was used with mouse antibodies raised to ASAH-1 (BD Biosciences: 612302, Wokingham, UK) and actin loading control. Visualisation and semi-quantitative analysis was performed to assess for the effects on AC expression with either ACsi or NTsi treatment.

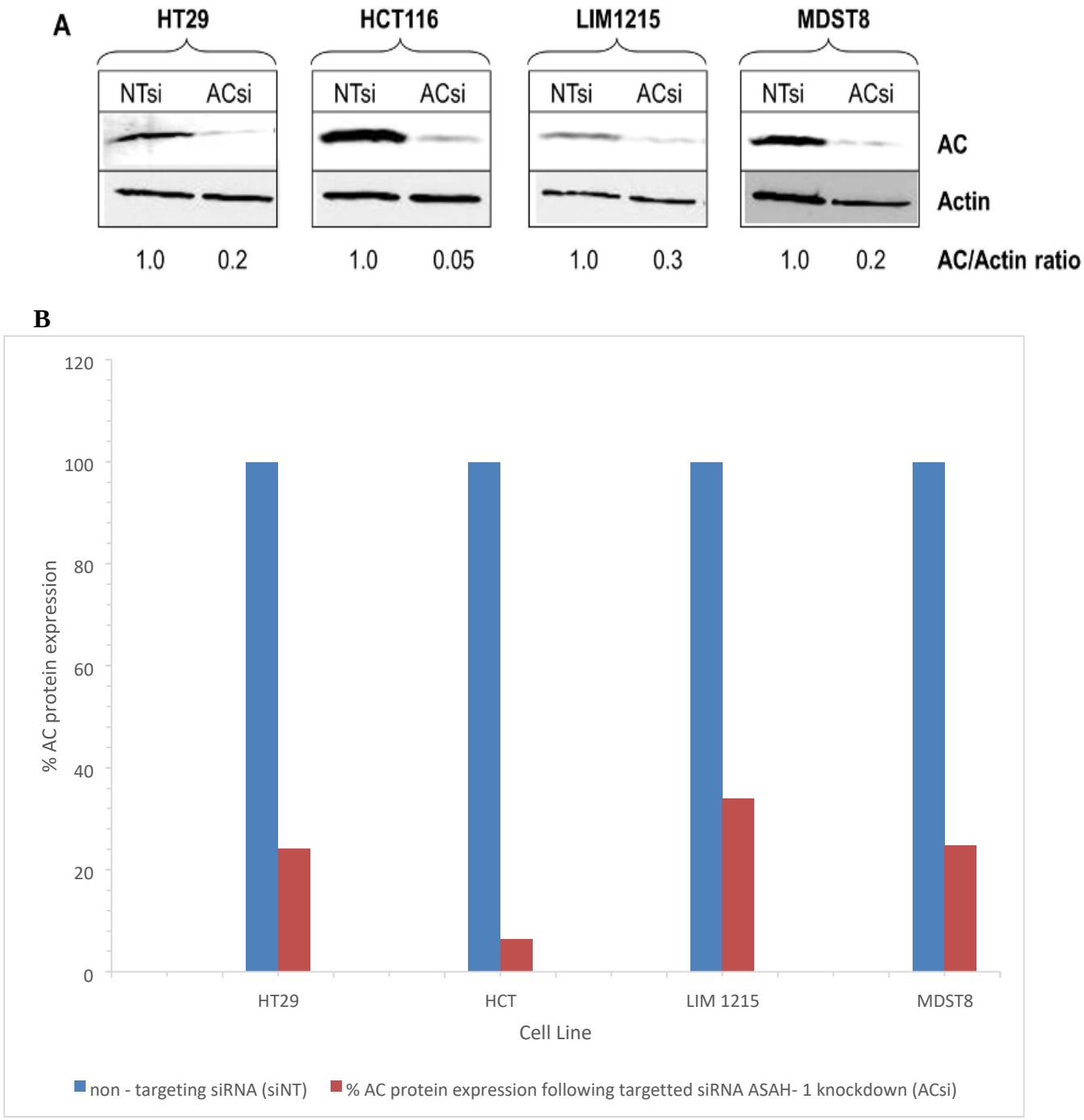


Figure 3.7 - Suppression of AC protein expression in CRC cells. HT29, HCT 116, LIM1215 and MDST8 cells were incubated for 48 h with non-targeting siRNA (NTsi) control vs siRNA ASAH-1 (ACsi). (A) Whole cell extracts were prepared and proteins were separated by SDSPAGE (16%) and analysed by immunoblotting using either AC or actin antibodies. Shown below each figure is the relative mean AC/actin ratio. Immunoblotting shows reduced AC expression for 4 Cell lines with non-targeting siRNA control vs siRNA AC. (B) Bar graph representation of immunoblotting data to confirm successful percentage AC knockdown with

targeted siRNA inhibition where NTsi expression of AC values were normalised to 100% with loading control with two repeats. The results show that through siRNA inhibition targeted against ASAH-1 there was a reduction in AC protein expression as determined through semi-quantitative analysis from immunoblotting. Statistical significance for siRNA AC knockdown was achieved across all cell lines (HT29 $p < 0.003$, HCT 116 $p < 0.0004$, LIM1215 $p < 0.013$, MDST8 $p < 0.014$).

HCT 116 cells displayed the most significant decrease in AC expression through siRNA inhibition with 93.6% reduction as compared to non-targeting control. HT29 and MDST8 cell lines showed a 75.9% reduction and 75.2% reduction in AC expression respectively. LIM1215 cell lines displayed a reduction of 66.1% AC protein expression. Therefore, across all cell lines there was an average of 77.7% reduction of AC expression through siRNA biological inhibition.

Microscopy photographs were taken of the cell lines at 48h post transfection which demonstrated that higher AC expressing cell lines (LIM 1215 and MDST8) appeared to display impaired growth when treated with siRNA AC (Chapter 2 : Materials and Methods 2.6.1).

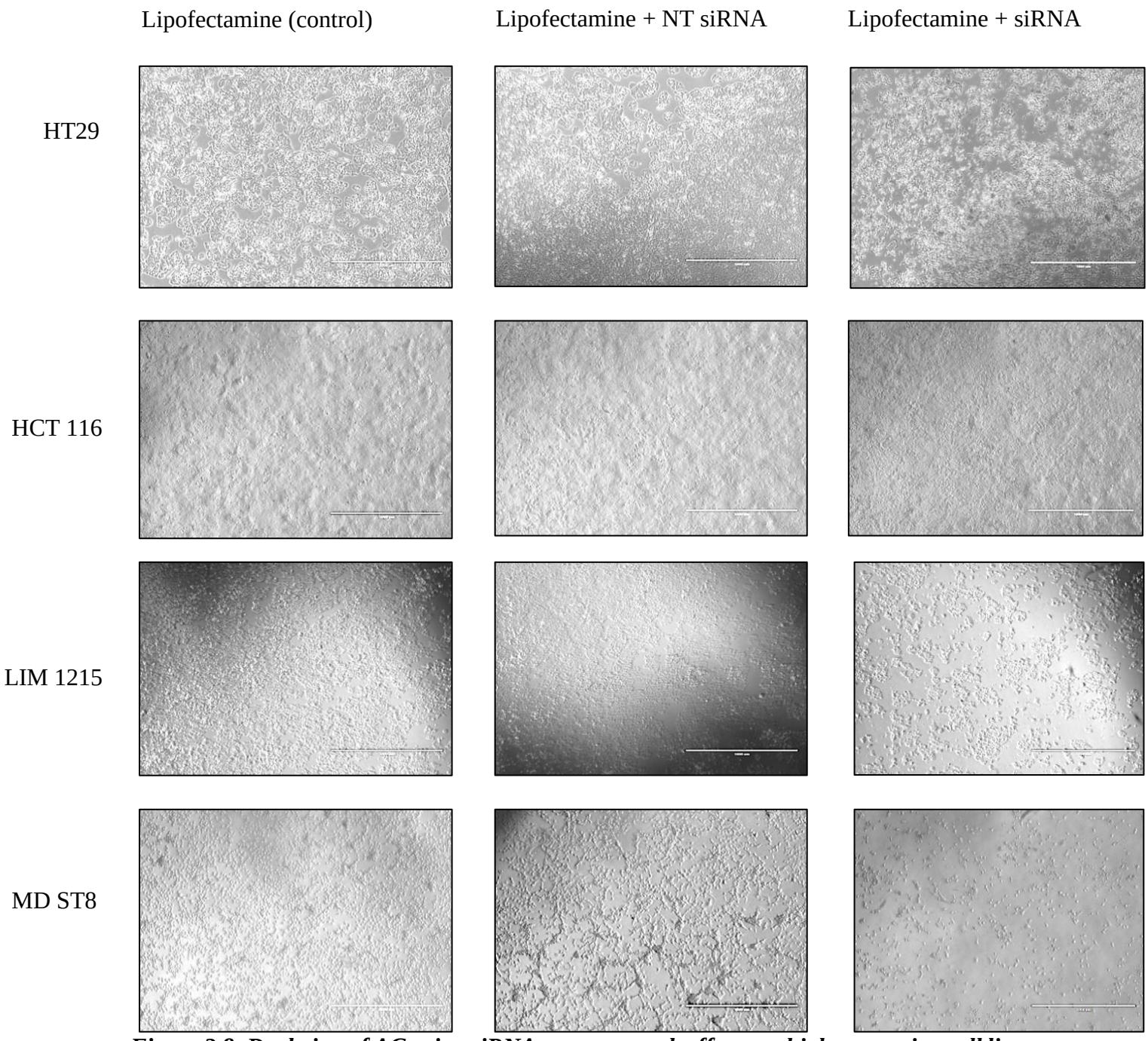


***Figure 3.8- Depletion of AC using siRNA causes growth effects on high expressing cell l*ines.**

These microscopy photographs of cell lines seeded onto 3cm dishes 48h post transfection with either lipofectamine only or lipofectamine and NT control siRNA or siRNA AC. 40nM siRNA (NT control and siRNA AC) was used to transfect HCT 116 and HT 29 cells as lower expressors and 80nM siRNA (NT control and siRNA AC) was used for LIM 1215 and MDST8 cell as high expressors.

From the microscope images, it can be noted that treatment with siRNA AC caused growth inhibition on the LIM 1215 and MDST8 cells as a result the MDST8 cells were not suitable for clonogenic survival assays with these sets of comparative treatments.

From this data which confirmed successful knockdown of AC expression, subsequent clonogenic survival assays would be developed using non-targeting control and siRNA AC for these cell lines to elucidate if each individual cell-line would display altered radiosensitivity with targeted AC inhibition.

3.5 Chapter results summary

Initial baseline immunoblotting across the six cell lines confirmed differential baseline AC protein expression using mouse antibodies raised against human ASAH-1 (BD Biosciences: 612302, Wokingham, UK). AC protein expression was normalised relative to HCT116, and that for example NC1 cells demonstrated ~10-fold less expression whereas MDST8 showed ~5.8-fold higher expression. These results therefore correlate with the data analysed from the initial panel of cells validated the results provided by the Sanger Institute.

The initial baseline clonogenic survival assays appeared to show that the cell lines displayed variable radiosensitivity at the set doses of x-ray treatment. The HCT-116 cells were generally more radiosensitive than the MDST8 and LIM1215 cell lines which were comparably more radioresistant. The results have shown that the higher baseline AC expressing cell lines appear to demonstrate a more radioresistant phenotype.

The results show that through siRNA inhibition targeted against ASAH-1 there was a reduction in AC protein expression as determined through semi-quantitative analysis from immunoblotting. Across all cell lines there was an average of 77.7% reduction of AC expression through siRNA biological inhibition.

Chapter 4 AC expression and radiosensitivity

4.1 Modulation of AC expression and its effect on radiosensitivity

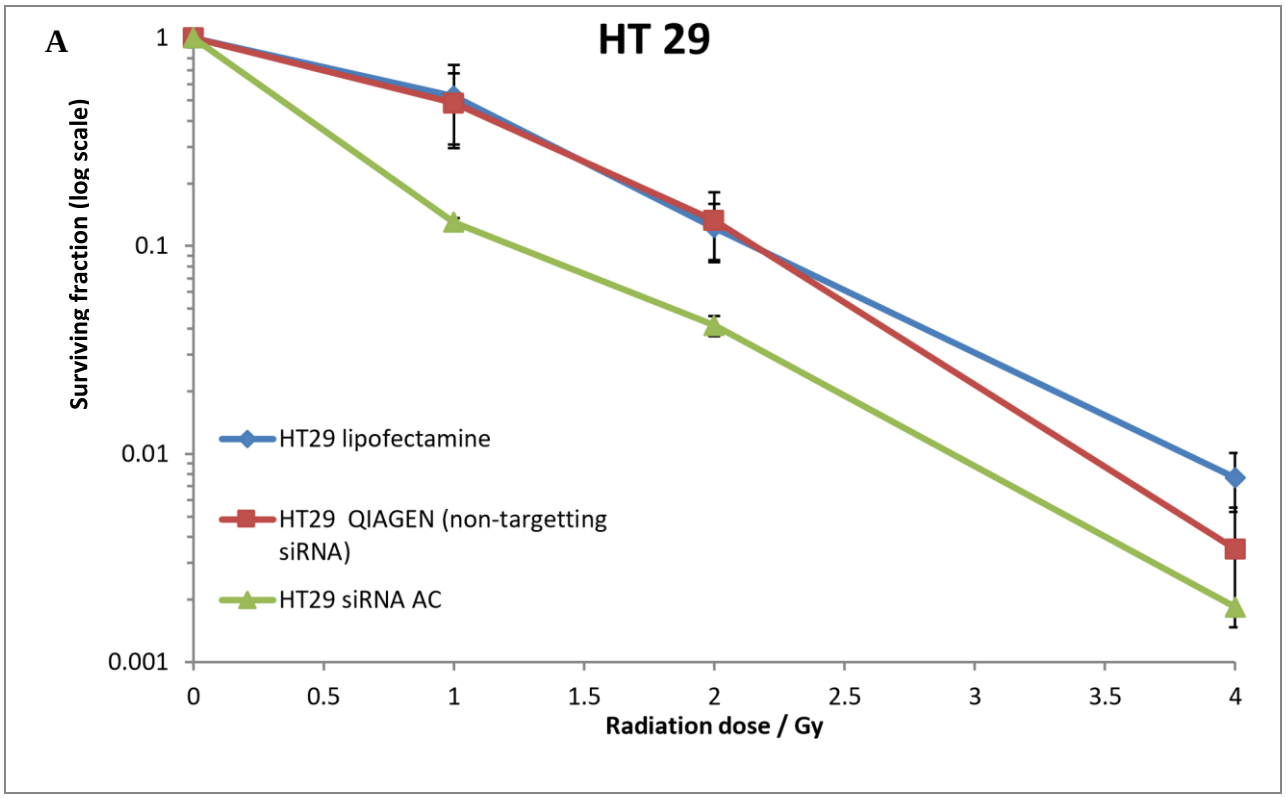
The most established evidence for the role of AC in controlling cell proliferation and radioresistance comes from prostate cancer. siRNA gene knockdown of AC in human PPC-1 cells and targeted drug inhibition with the small molecule inhibitor LCL385 conferred improved radiosensitivity, and conversely upregulation of AC expression conferred increased radioresistance *in vitro* (Mahdy *et al.*, 2009). Increased AC expression has been linked to poorer tumour response in multiple different cancers including head and neck, myeloid leukaemia, melanoma, hepatobiliary cancers and glioblastoma.

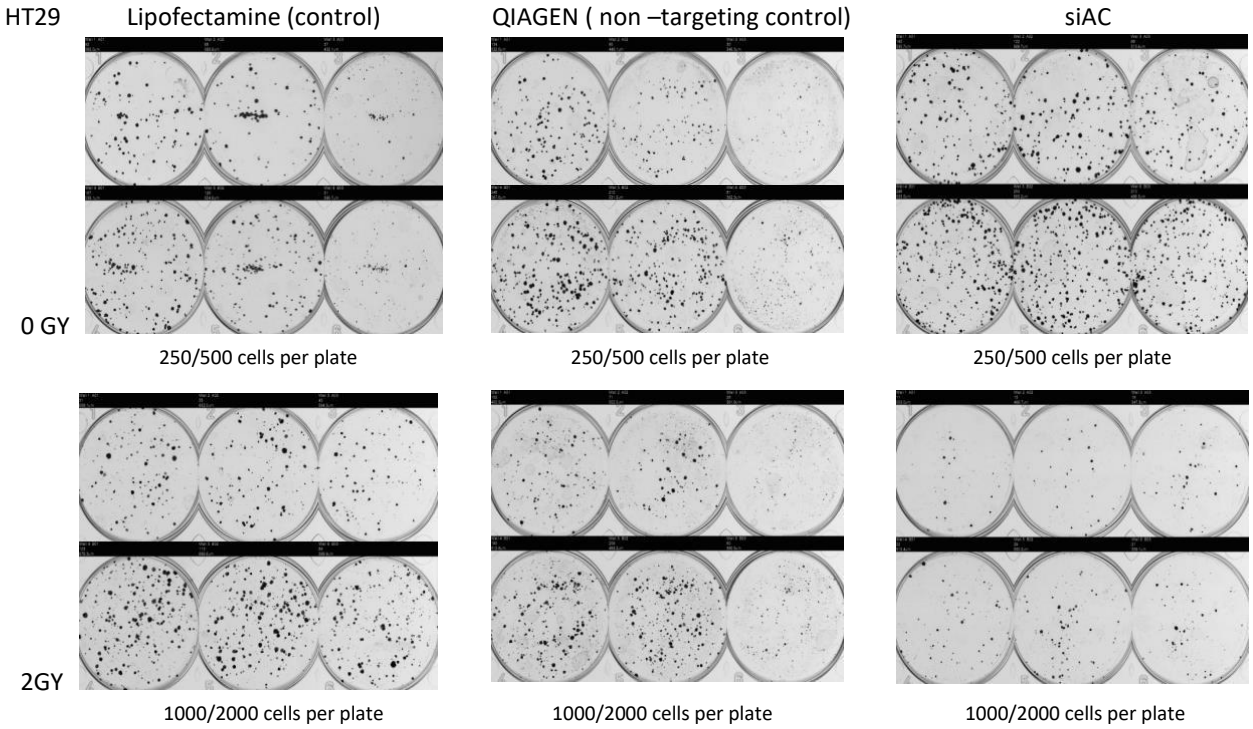
Increased AC expression has been demonstrated in colon cancer cells, and confirmed on IHC compared with normal colonic tissue, and inhibiting AC shown to sensitize cells to oxaliplatin (Klobučar *et al.*, 2018). Other studies have also shown that through inhibiting AC there is increased apoptosis (Baspinar *et al.*, 2017). As described in the previous work from our research group, proteomic and IHC analysis showed that AC expression was associated with poorer CRT response (Bowden *et al.*, 2018).

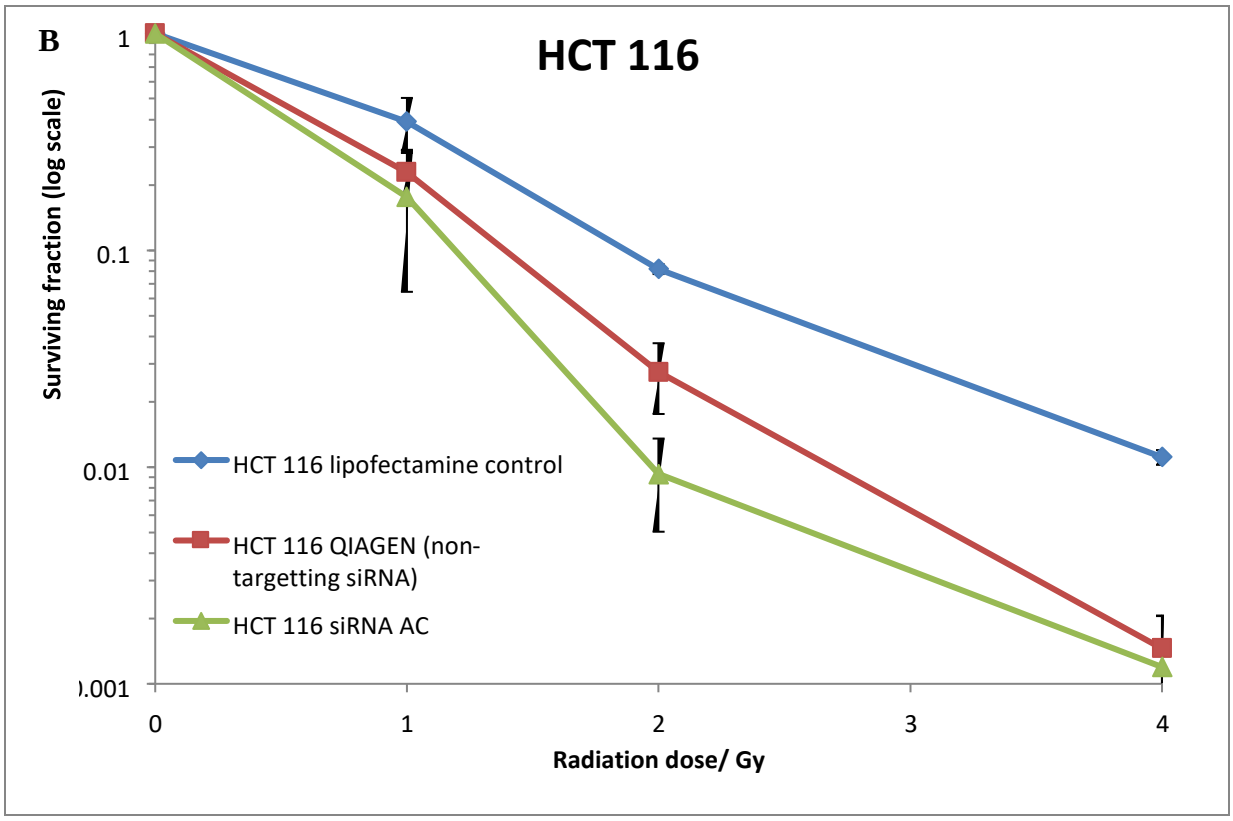
Experimental question:

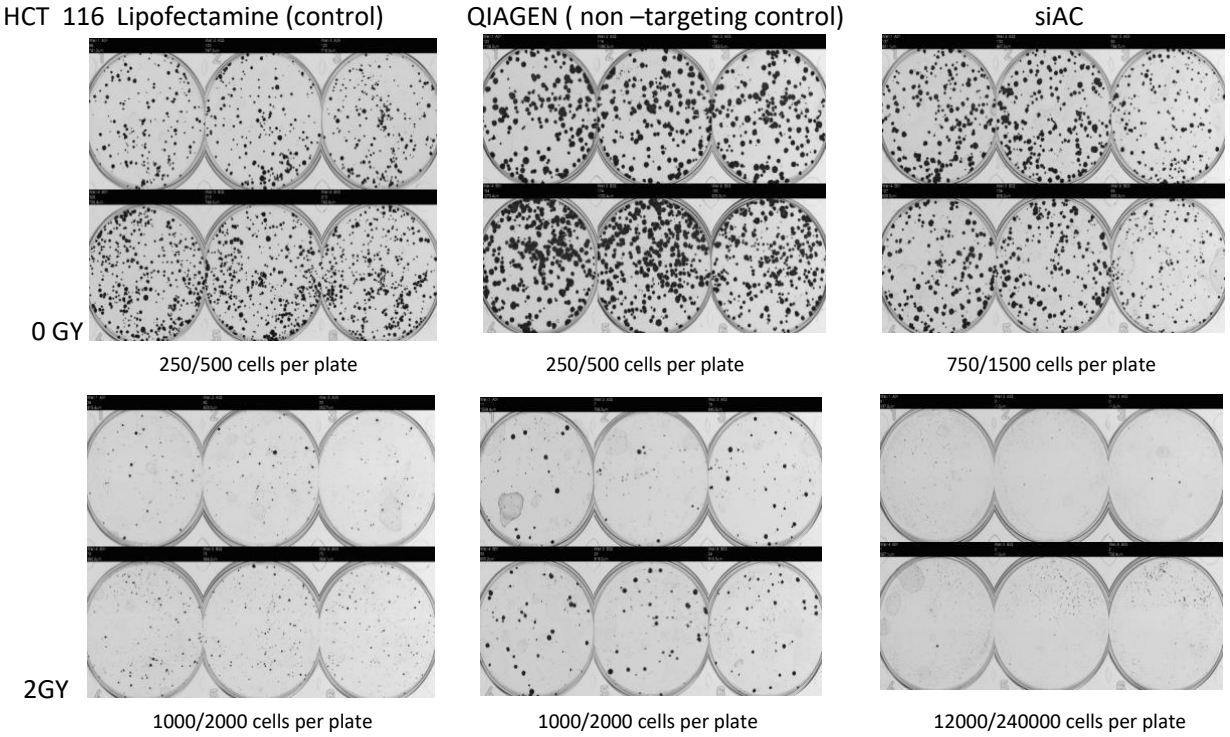
Does manipulation of AC expression alter the radiosensitivity of CRC cell lines?

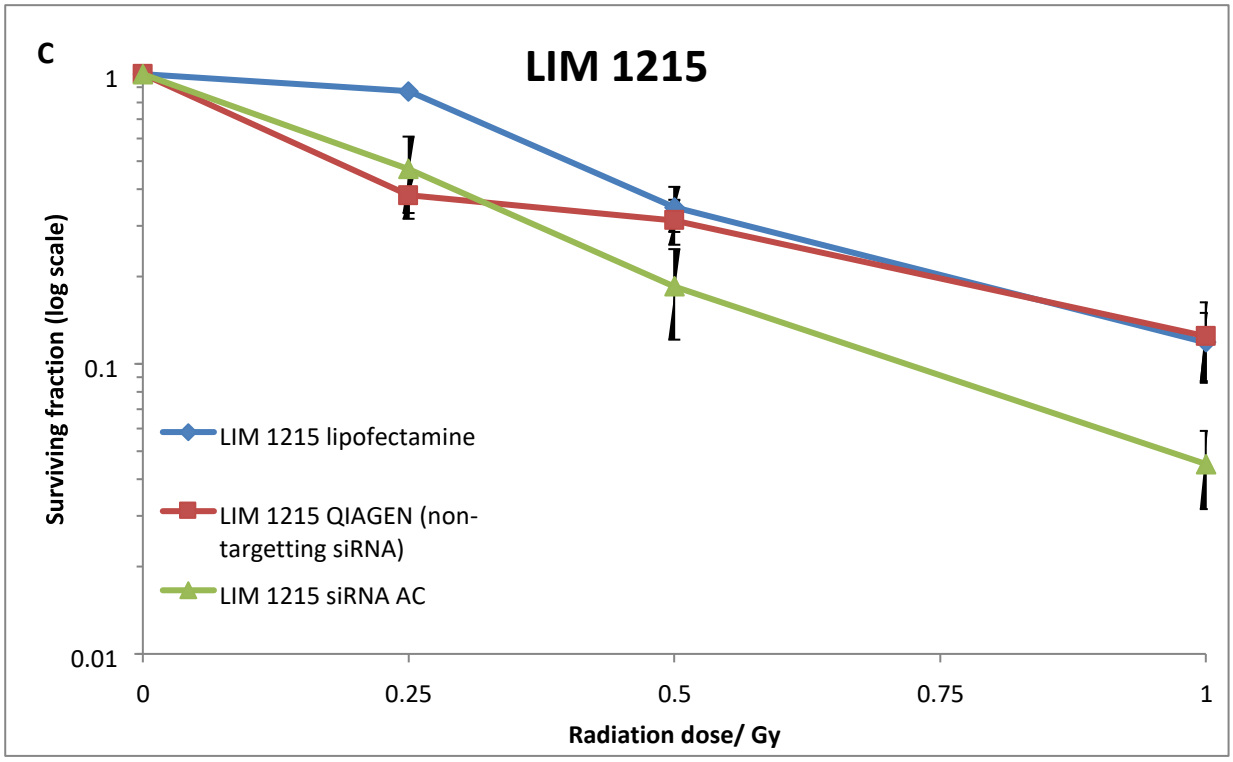
4.2 Clonogenic survival assays with targeted siRNA AC






B
HCT 116
Surviving fraction (log scale)
1
0.1
0.01
.001
0
1
2
3
4
Radiation dose/ Gy
HCT 116 lipofectamine control
HCT 116 QIAGEN (non-targetting siRNA)
HCT 116 siRNA AC


HCT 116 Lipofectamine (control)
QIAGEN (non –targeting control)
siAC
0 GY
250/500 cells per plate
250/500 cells per plate
750/1500 cells per plate
2GY
1000/2000 cells per plate
1000/2000 cells per plate
12000/240000 cells per plate


C
LIM 1215
1
0.1
0.01
Surviving fraction (log scale)
LIM 1215 lipofectamine
LIM 1215 QIAGEN (non-targetting siRNA)
LIM 1215 siRNA AC
0
0.25
0.5
0.75
1
Radiation dose/ Gy

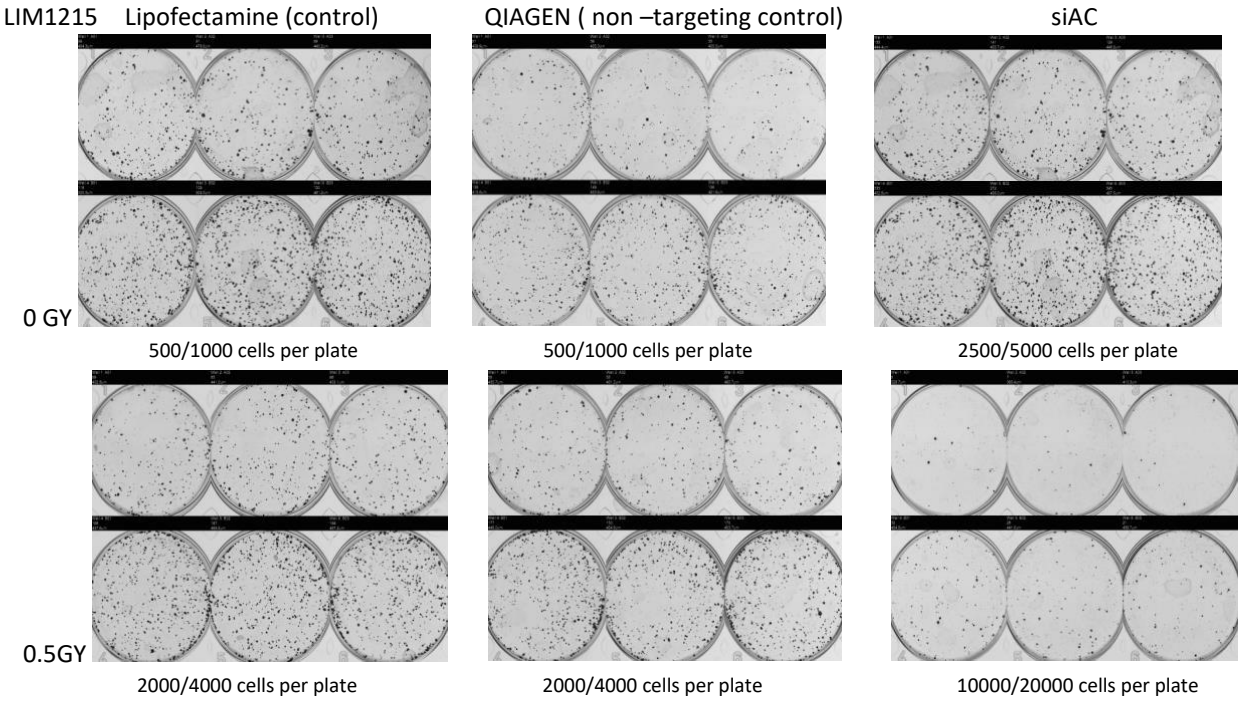

LIM1215
Lipofectamine (control)
QIAGEN (non –targeting control)
siAC
0 GY
500/1000 cells per plate
500/1000 cells per plate
2500/5000 cells per plate
0.5GY
2000/4000 cells per plate
2000/4000 cells per plate
10000/20000 cells per plate

Figure 4.1 - Suppression of AC correlates with increased radiosensitivity of CRC cells, clonogenic curves and representative colony plate photographs. (A) HT29, (B HCT 116 or (C) LIM 1215 cells were treated with Lipofectamine transfection reagent alone, or in combination with non-targeting control (AllStars Negative Control siRNA; Qiagen, Manchester, UK) or siRNA AC (D-005228-03, Horizon Discovery Ltd, Cambridge, UK). Shown is the mean surviving fraction ± S.E. from three independent experiments.

The clonogenic survival assays for the HT29 cells there were fewer surviving fractions in the cells treated with siRNA AC compared to non-targeting and lipofectamine controls at 1, 2 and Gy respectively. The curves separated at 2Gy and the results of non-targeting control *vs* siRNA AC were significant for reduced radiosensitivity with AC depletion ($p<0.00004$). Below the survival curves the representative clonogenic plates further illustrate these data.

For HCT 116 cells the clonogenic survival assay showed the same findings with the cell line displaying increased radiosensitivity with AC depletion at 1,2 and 4 Gy respectively, particularly evident at 2Gy. The results were similarly significant with non-targeting control *vs* siRNA AC ($p<0.03$). The representative clonogenic plate data represents the data generated to plot the survival curves. Of note NT control siRNA also appeared to have an impact on cell survival post-irradiation in HCT116 cells, the reasons for this are unclear, however statistical significance was achieved when comparing NT control siRNA *vs* siRNA AC.

In the LIM 1215 cell line separation of each curve at 0.5Gy was evident with overall increased radiosensitivity noted in the AC depleted cells in keeping with the findings from the other cell lines. The treatment dose and seeding numbers were altered for the LIM 1215 as these cells may rely heavily on AC for growth and proliferation. Statistical significance as again achieved with non-targeting control *vs* siRNA AC ($p<0.001$).

The MDST8 cells were not used for these clonogenic survival assays because when treated with AC siRNA they did not grow into effective colonies post irradiation that could be counted, therefore the results obtained were not suitable for analysis. The underlying mechanism behind this is not fully understood, however we have postulated that the higher expressing cell lines such as the MDST9 and the LIM 1215 cells are heavily reliant on AC for cell growth and proliferation, making it difficult to analyse radiosensitivity.

Note that the NT control siRNA also appeared to have an impact on cell survival postirradiation in HCT116 cells, the reasons for this are unclear, however statistical significance was achieved when comparing NT control siRNA *vs* siRNA AC.

Comparative treatment	**HCT 116**	**HT 29**	**LIM 1215**
NT siRNA *vs* AC siRNA	$p<0.03$	$p<0.00004$	$p<0.001$
Lipo *vs* AC siRNA	$p<0.68$	$p<0.002$	$p<0.05$

Table 4.1 *- Statistical significance on colony survival post irradiation with cells treated with siRNA AC vs NT control using "CFAssay for R" software. This table summarises the statistical analysis for the comparative treatments in all three cell lines.*

In addition, note that the x-ray doses for the LIM 1215 cells were lowered from 0-1Gy, this was due to siRNA AC treatment causing impaired growth effects on these cells and inaccurate colony counts at standard dosing. These cell lines were included for subsequent and experiments for analysis as the colonies yielded at the modified dosing were suitable for analysis.

There is sufficient evidence to support the role of AC in cell growth and proliferation within the literature and we have postulated that certain cells may express AC at higher concentrations as they are reliant on it as a cell-growth factor, however this may need to be investigated further specifically in these CRC cell lines.

4.3 Chapter results summary

The clonogenic survival curves illustrated that through biological inhibition of AC, all cell lines displayed increased radiosensitivity. The results of these all achieved statistical significance. This implicates the role of AC expression in radiosensitivity within CRC cell lines therefore showing similar results seen in other cancers from the literature.

Chapter 5 Mechanistic work

5.1 Elucidating a mechanism linking AC expression with post-irradiation apoptosis

5.1.1 Ceramide and apoptosis

Ceramide can accumulate intracellularly in response to stress which can cause cell death *(See Figure 1.8 adapted from* Shubhra Chakrabarti *et al.*, 2016) .It was first shown in leukaemia cells that accumulation of ceramide caused cell death through apoptosis (Obeid *et al.*, 1993). Ceramide accumulation in mitochondria induces the pro-apoptotic protein Bax to become recruited, which subsequently activates the caspase pathway and ultimately apoptosis (Chipuk *et al.*, 2012). Ceramide also acts as a second messenger of the apoptotic cascade via CD95 (Grassmé, Schwarz and Gulbins, 2001) and interaction with nitric oxide to cause cell death (Takeda *et al.*, 1999). Ceramide has also been shown to reduce telomerase activity therefore causing telomere shortening, accelerated senescence and apoptosis in lung cancer cells (Ogretmen *et al.*, 2001).

Many cancer cells have developed methods to extract ceramide from the cell (Truman *et al.*, 2014) in conjunction with upregulation of ceramidase enzymes to metabolise pro-apoptotic ceramide into pro-survival sphingolipids (Patmanathan *et al.*, 2017).

AC itself has been implicated specifically in the development of cancer through two mechanisms:

1. Identification of its over expression in human cancer and/or relationship to stage or prognosis.
2. Observation that its inhibition and consequent rise in ceramide levels leads to apoptotic cell death.

There is a complex interplay between pro-apoptotic ceramide and pro-survival S1P (See figure 1.9 taken from Govindarajah *et al.*, 2019). This is a dynamic system where AC is a crucial enzyme in this pathway that converts ceramide into sphingosine. From this pathway there are several areas that could be manipulated in order to change the balance between cell death and cell survival.

Poly (ADP-ribose) polymerase-1 (PARP-1)

PARP-1 is a key enzyme that plays a crucial and complex role in multiple DNA repair pathways in an NAD + dependent process involving other proteins such as XRCC-1, ligase III and Polß and is in effect a "first responder" to DNA damage (Pascal, 2018).

This ability for PARP-1 to recognise and repair complex DNA damage lesions has been determined as clinically important in several cancers. Over the past decade, several commercially available PARP inhibitors have been utilised in the treatment of breast and ovarian cancer (Mateo *et al.*, 2019).

PARP-1 itself is also a key substrate of the caspases (3/7) and during apoptosis, PARP-1 itself is cleaved into highly specific and detectable fragments. PARP-1 cleavage by these caspases results in the production of 2 specific fragments: an 89-kD catalytic fragment and a 24-kD fragment (Margolin *et al.*, 1997), therefore through the detection and quantification of these fragments it can be a surrogate marker of apoptotic activity within cells.

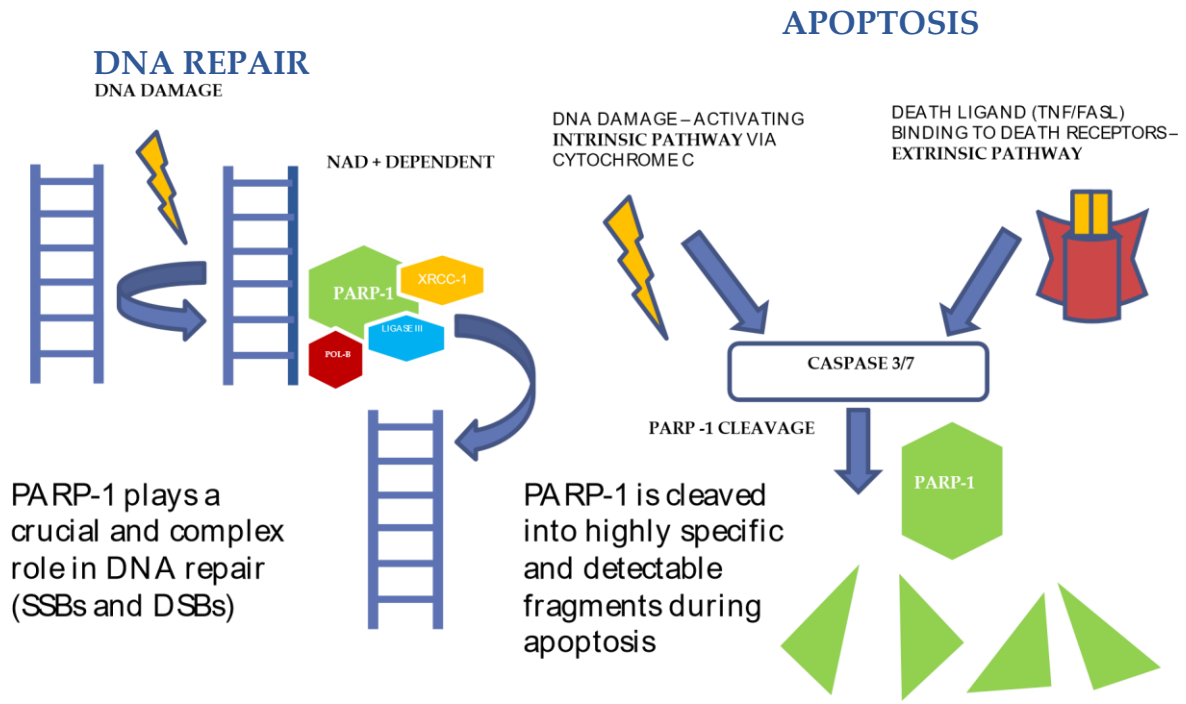


Figure 5.3 – ***The role of PARP-1.*** Diagram illustrating the role of PARP-1 in both DNA repair and also as a specific marker of apoptosis.

Experimental question:

If AC expression correlates with CRC cell lines radioresistance, can we elucidate a potential mechanism?

5.2 Poly (ADP-ribose) polymerase-1 (PARP-1) immunoblotting analysis

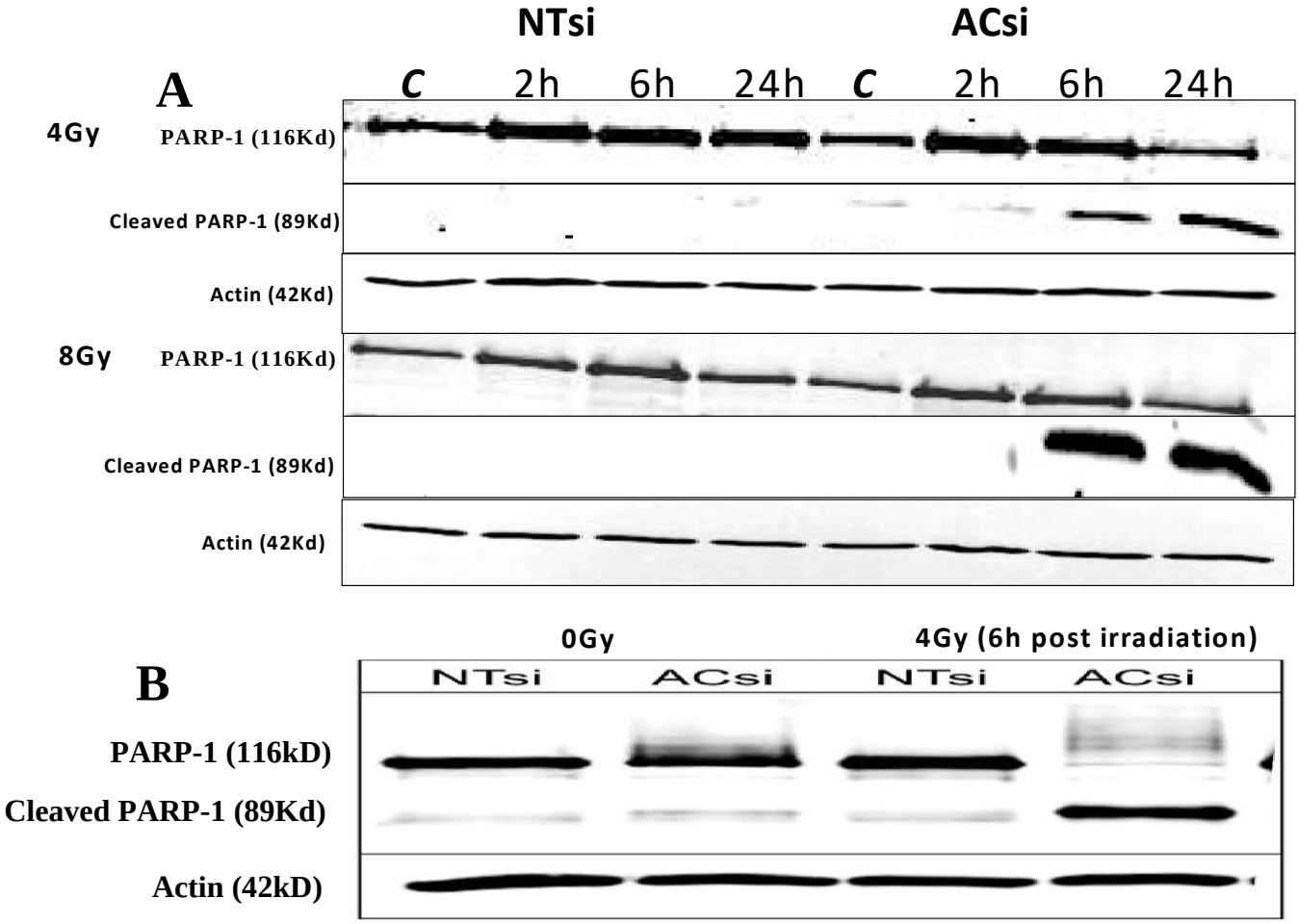
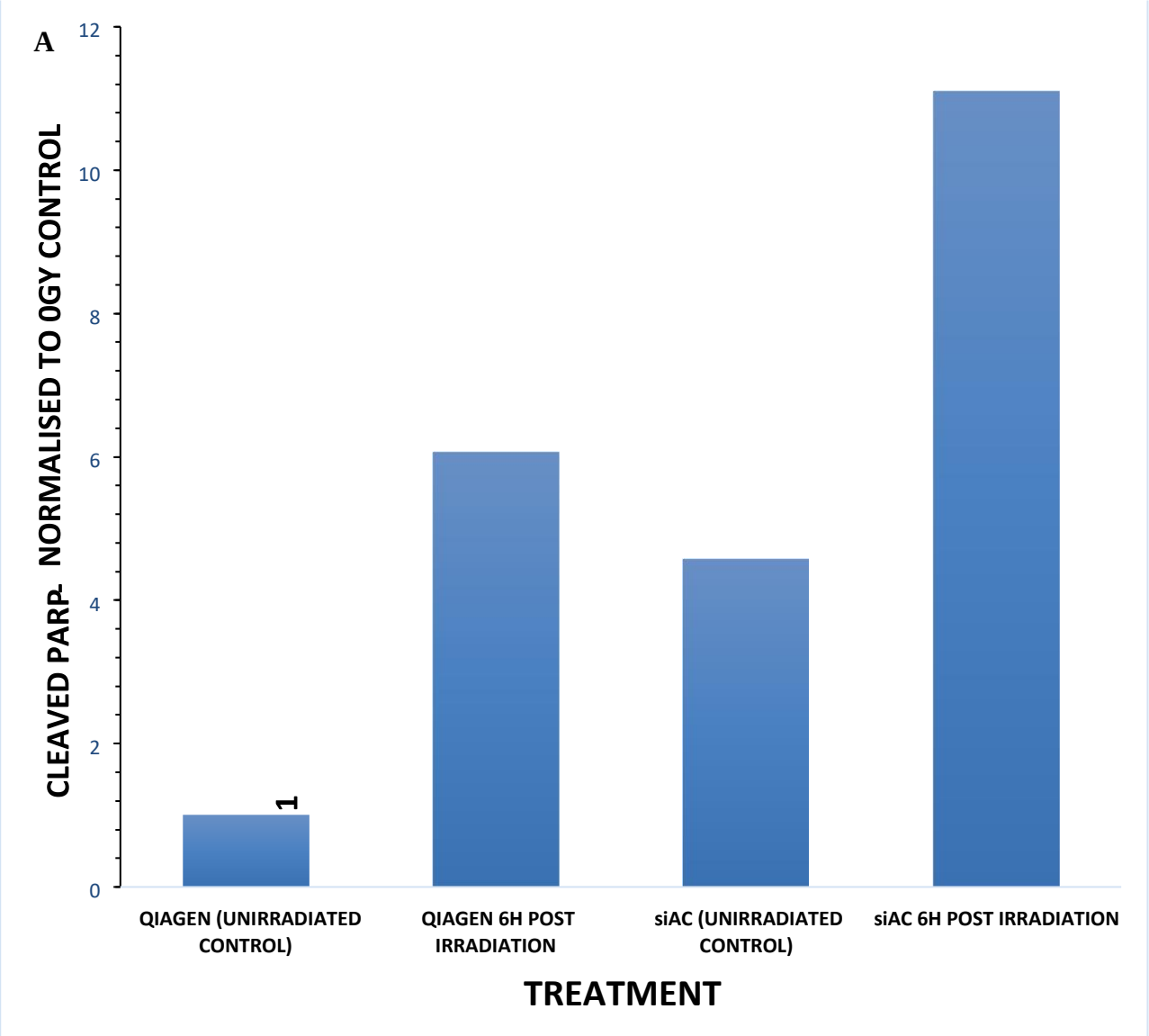


Figure 5.4 – ***Immunoblotting data to show increased apoptosis in AC depleted HT 29 and HCT 116 cells in response to irradiation.*** (A) HT 29 cells were treated with non-targeting control (NTsi) or AC siRNA (ACsi) for 48 h, and either unirradiated control (C) or irradiated with 4 or 8 Gy x-rays and incubated for 2, 6 and 24h timepoints at 37°C. Whole cell extracts were prepared, and proteins separated and analysed accordingly. (B) HCT116 cells were treated with non-targeting control (NTsi) or AC siRNA (ACsi) for 48 h, and either unirradiated or irradiated with 4 Gy x-rays and incubated for 6 h at 37°C. Whole cell extracts were prepared and proteins were separated by 10% SDS-PAGE and analysed by immunoblotting using either PARP-1 or actin antibodies note the full length PARP 1 (116Kd) has almost entirely been cleaved into the 89Kd fragment.

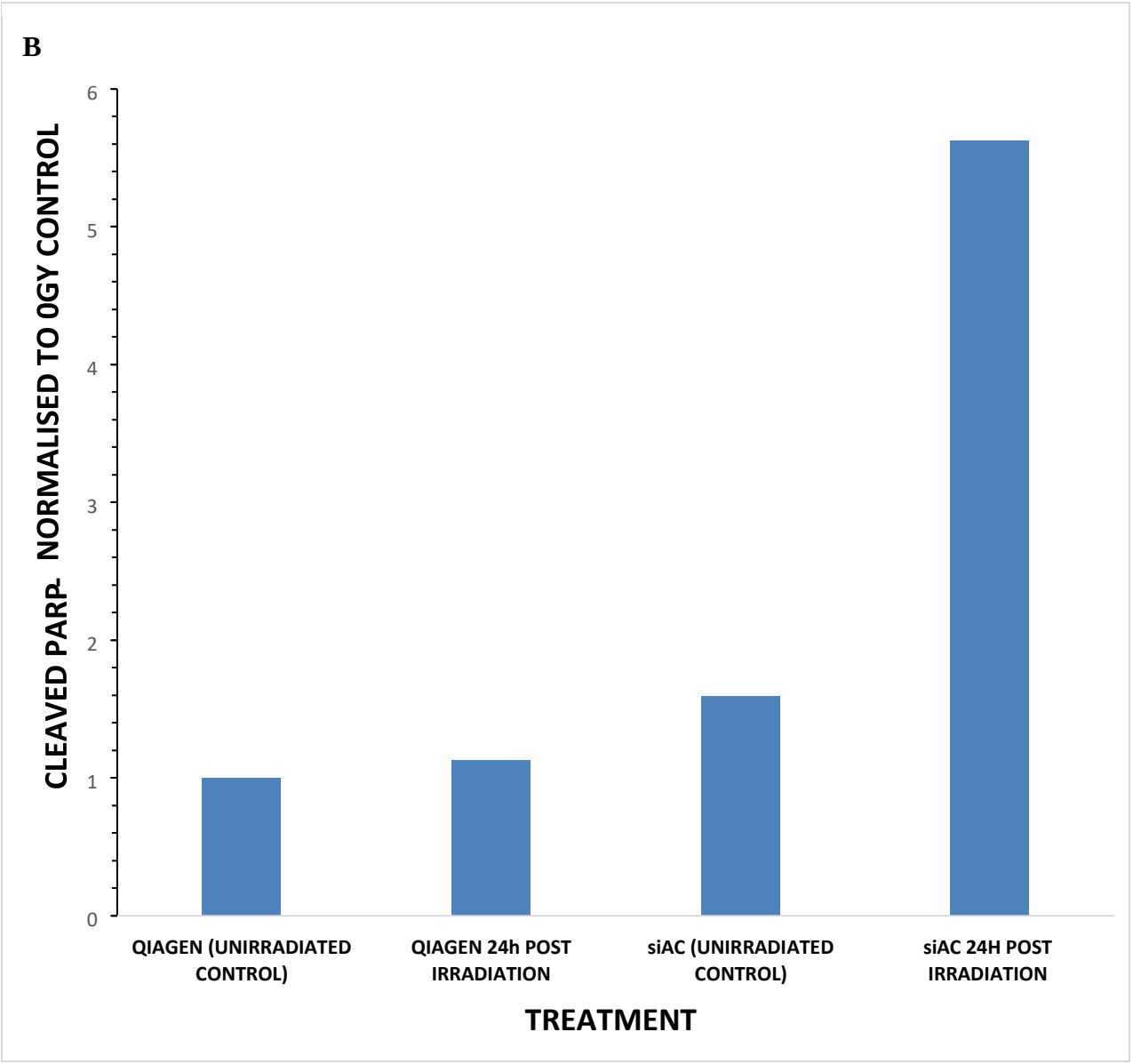
Immunoblotting of WCE from both HT 29 and HCT 116 cells has shown that in cells depleted with AC using siRNA displayed significantly higher levels of cleaved PARP-1 post irradiation at multiple time points and different doses of x-ray irradiation. For HCT 116 cells at 6h post 4Gy x-ray irradiation the full-length PARP -1 has almost been entirely cleaved into its smaller 89Kd fragment. In both cell lines there was no significant PARP cleavage within the NTsi treatment samples. In addition, with HT 29 cells there is a visible difference in the size and intensity of cleaved PARP-1 at 8Gy compared to 4Gy dosing, indicating higher levels of apoptosis.

This data suggests that depletion of AC causes decreased cell survival in response to radiation through the apoptotic pathway as PARP-1 is cleaved into specific detectable fragments during apoptosis and quantifying these levels is a validated technique to detect apoptosis.

5.3 Quantification of PARP-1 cleavage through immunoblotting data



B


CLEAVED PARP NORMALISED TO 0GY CONTROL
0
1
2
3
4
5
6
QIAGEN (UNIRRADIATED CONTROL)
QIAGEN 24h POST IRRADIATION
siAC (UNIRRADIATED CONTROL)
siAC 24H POST IRRADIATION
TREATMENT

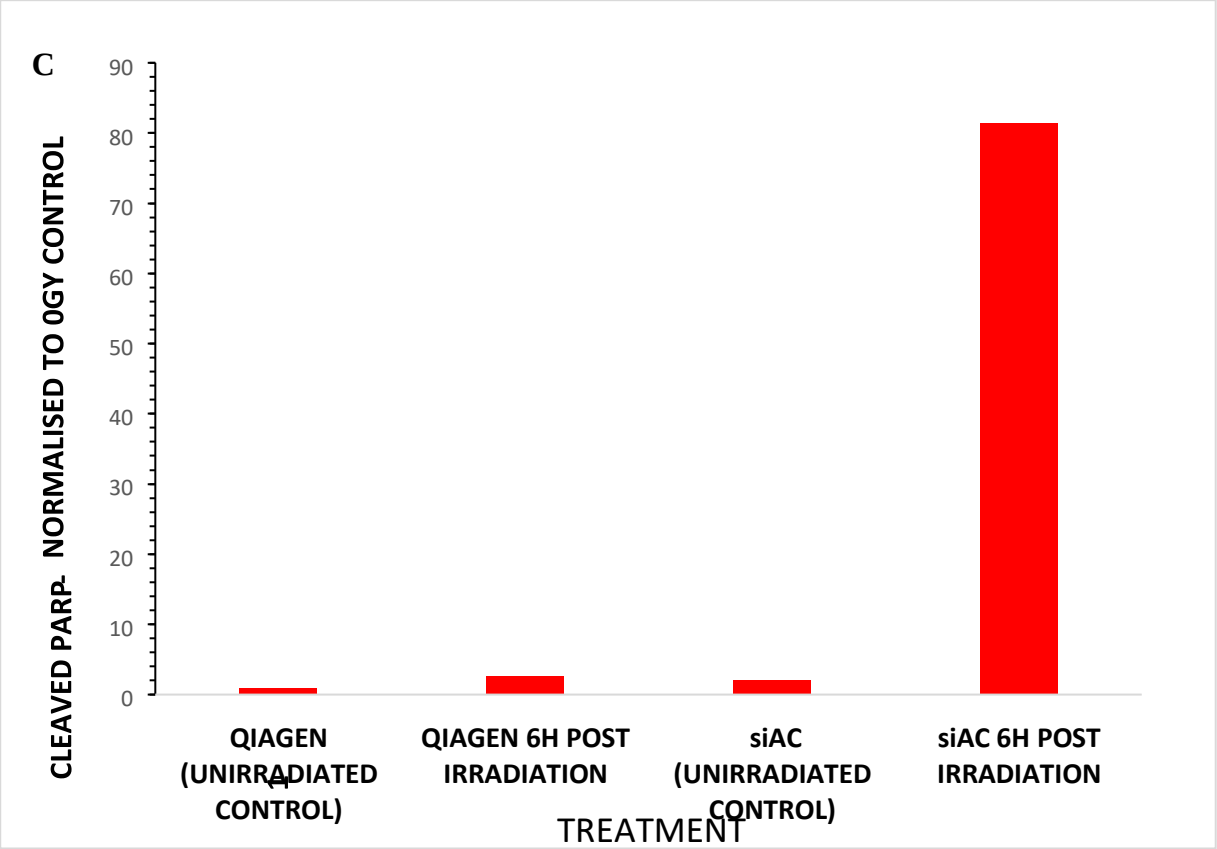


Figure 5.5 - ***Bar chart to illustrate quantitative increase in apoptosis in AC depleted cells post irradiation***. Expression normalised to unirradiated control (shown above are results from three independent repeat experiments). (A) HT 29 cells were treated with non-targeting control (QIAGEN) or siRNA AC for 48h, and either unirradiated or irradiated with 4Gy x-rays and incubated for 24 h at 37°C. Cleaved PARP – 1 levels were higher in AC depleted cells 6h post irradiation ($p < 0.05$ siRNA AC (0.01) *vs* NT control (0.006)). (B) WCE from HT 29 cells 24h post 4Gy irradiation show much higher PARP-1 levels indicating higher levels of apoptosis compared with control ($p = 0.3$ siRNA AC (0.27) *vs* NT control (0.05)). (C) HCT116 cells were treated with non-targeting control (QIAGEN) or siRNA AC (siAC) for 48 h, and either unirradiated or irradiated with 4Gy dose and incubated for 6 h at 37°C. Cleaved PARP – 1 levels were higher in AC depleted cells 6h post irradiation ($p < 0.01$ siRNA AC (0.16) *vs* NT control (0.005).

For this analysis PARP-1 cleavage levels were normalised to 0Gy unirradiated control and the relative amount of PARP-1 was detected through semi-quantitative immunoblotting results, statistical analysis was performed using a t test.

HT29 cells displayed higher levels of apoptosis in AC depleted cells at both 6 and 24h post irradiation compared to non-targeting control. 6h post irradiation there was 1.8- fold increase with siRNA AC *vs* NT control $p < 0.05$, at 24h this difference was more pronounced with a 5fold increase in cleaved PARP-1, however this result did not achieve statistical significance ($p = 0.3$).

For HCT 116 cells similar effects were observed with AC depleted cells displaying higher levels of apoptosis compared to non-targeting control, at 6h post irradiation there was a 30-fold increase in cleaved PARP – 1 as a surrogate marker of apoptosis ($p < 0.01$).

The results suggest that AC expression could play a role in preventing apoptosis secondary to DNA damage from ionising radiation in these CRC cell lines.

The observed difference in inducible apoptosis in the AC depleted cells for both cell lines could be due to numerous factors however as detailed previously, the HCT 116 cells are p53 +/+ compared to the HT 29 cells which are p53 -/-. This may have a function to play as p53 plays an important role in the induction and propagation of apoptosis from ionising radiation.

5.4 Chapter results summary

These graphs of the immunoblotting data illustrated higher cleaved PARP-1 levels post irradiation in both HT29 and HCT 116 cell lines. As PARP-1 is a specific and detectable marker of apoptosis, we have postulated that in these CRC cell lines, AC expression may mediate radioresistance through altered apoptosis post -irradiation.

Chapter 6 Discussion

6.1 Background

Colorectal cancer is the second most common cause of cancer-related death in the UK with rectal cancer specifically accounting for approximately 25% of these cases. Surgical resection remains the gold standard treatment, however neoadjuvant CRT remains the mainstay of treatment for locally advanced rectal cancer (Li *et al.*, 2016) and it is often utilised in selected patients with locally advanced disease to downstage tumours and improve the probability of clear surgical margins. The treatment response to neoadjuvant CRT remains both variable and unpredictable, leading to a drive to develop predictive biomarkers of radiotherapy response and potential radiosensitisers for clinical use (Dayde *et al.*, 2017). Favourable CRT response not only allows surgical resection of the tumour with clear margins, but if the pathological response is significant there is an associated improvement in overall long-term survival. Only approximately 12 % of patients with locally advanced rectal cancer experience a complete pathological response (Martin, Heneghan and Winter, 2012) with a further proportion of patients developing disease progression (locally or systemically) whilst on therapy.

6.2 Previous work

Our research group has previously employed proteomic profiling in conjunction with immunohistochemical validation to identify a novel finding that AC is associated with poor response to CRT in patients with advanced rectal cancer. This initial work analysed the AC expression in pre-treatment biopsies of patients with subsequent validation work analysing only surgical resection specimens, therefore its efficacy as a predictive biomarker of response remains unknown.

This study followed on from this previous work that utilised proteomic and

immunohistochemical analysis has identified that high levels of acid AC expression confers poorer response to neoadjuvant treatment in locally advanced rectal cancer. Several proteins were identified from serial biopsies of patients with LARC that correlated with poorer response to neoadjuvant radiotherapy. From these candidate proteins, AC was identified as a protein of interest as its expression correlated with poorer tumour responses to pre-operative radiotherapy. AC has been implicated in the pathogenesis of several other cancers and its manipulation in head and neck, central nervous system (CNS) and prostate cancer was shown to have direct effects on chemo and radiosensitivity through pro-apoptotic pathways (Mahdy *et al.*, 2009) (Govindarajah *et al.*, 2019) (Bedia *et al.*, 2011) (Nguyen *et al.*, 2018). The implication of these findings is that AC might play a role in the response of tumour cells to ionizing radiation but its role in the radiotherapy response of rectal cancer is yet to be fully studied and understood.

From the preceding work, the aims of this were firstly to validate the Sanger Institute data and establish differential AC expression across a panel of CRC cell lines. Then to modulate AC expression to assess if this had any effect on radioresistance and if this was proven, to elucidate a potential mechanism.

6.3 Baseline AC expression in a panel of CRC cell lines

A panel of CRC cell lines (NCI H716, GEO, HCT 116, HT 29, LIM 1215, MDST8) was selected from an initial screened panel of 49 CRC immortalised cell lines performed at the Sanger Institute, categorised by their respective levels of ASAH-1 mRNA expression. Our research group had already analysed and modulated AC expression in HCT 116 p53 +/+ cells, therefore these cells were used as a validated comparator for the selected CRC cell lines (Bowden et al., 2018).

Immunoblotting and quantification of AC expression confirmed that in a panel of CRC cell lines there is indeed variable baseline AC expression. AC protein expression was normalised relative to HCT116, and that for example NC1 cells demonstrated ~10-fold less expression

whereas MDST8 showed ~5.8-fold higher expression. Subsequent clonogenic survival assays confirmed that AC expression correlated with baseline radioresistance in a panel of four cell lines that were suitable to undergo this assay. LIM 1215 and MDST8 cells, displayed higher colony survival at increased doses of x-ray irradiation compared to HCT 1116 cells that were more radiosensitive.

AC expression was determined using immunoblotting with the ASAH-1 antibody (BD Biosciences: 612302, Wokingham, UK), this specifically detected the active α-subunit (13-14Kd). AC itself is initially made as a larger pre-cursor protein (55kD) prior to being cleaved into the α (13-14Kd) and β (40kD) and once synthesized there is a complex method of transport into the Golgi apparatus where post translational modification occurs including glycosylation. During this process, AC is also cleaved into the active subunit and then transported into the lysosome however the nature of the protease involved for breakdown of AC and its method of transport into the lysosome remains unknown (Shtraizent *et al.*, 2008). This unknown mechanism of AC lysosomal transport and cleavage is interesting as this may occur differently across varying cell lines therefore giving different apparent AC expression phenotypes. Additionally, if the protease involved in AC cleavage from precursor protein into active protein can be identified it could offer another method of potential inhibition.

As detailed previously, the reasons for the difference in baseline radiosensitivity could be due to multiple factors including stage in cell cycle, passage number of cells or inherent radiosensitivity phenotypes independent from AC expression. Different tumour cells have different abilities to sustain DNA damage, particularly DSB's from ionizing radiation and it is this initial induction of DSBs and subsequent ability to repair or undergo cell death that is an important determinant of intrinsic radioresistance (El-Awady et al., 2003). This relationship between AC and radiosensitivity did not necessary imply causality, hence the progression of this work into targeted AC inhibition with siRNA.

6.4 siRNA inhibition of AC in CRC cell lines

In this study, siRNA depletion of AC was performed using a single oligonucleotide sequence for enhanced specificity. This sequence was identified from a pool of initial oligonucleotides in previous work by our research group. In these selected cell lines, following transfection with siRNA AC and non-targeting control mRNA, immunoblotting experiments confirmed that AC expression was reduced by approximately 70% across four different cell lines (HT 29, HCT 116, LIM 1215 and MDST8) in siRNA AC *vs* non-targeting control.

For the higher AC expressing cell lines, higher doses of siRNA AC were used to transfect the cells compared to lower expressing cell lines to ensure significant reduction in AC protein expression across all cell lines.

Following siRNA depletion of AC, it was noted that there were effects on cell growth and viability, in particular the high AC expressing MDST8 cell lines. Following depletion of AC there were higher numbers of these cells that had not formed adherent scaffolds with neighbouring cells and some had become rounded and detached from the surface of the tissue culture plastic. This indicated that some of the cells had died following transfection with siAC. It remains unclear if these cells over-express AC as it is relied upon as an important cell growth factor and regulator. Many cancers namely prostate, melanoma and glioblastoma overexpress AC as part of their pathophysiology, as through AC over-expression the sphingosine rheostat is shifted towards anti-apoptotic S1-P production as opposed to pro-apoptotic ceramide production (Doan et al., 2017; Hannun & Obeid, 2008; Obeid et al., 1993). In CRC cell lines the relative over expression of AC and its associated effects on tumour behaviour still not fully understood.

6.5 AC depletion and radioresistance

Clonogenic assays formed a significant part of the methodology within this study, as they are the gold standard for measuring cell survival post-irradiation. Once successful transfection with siRNA AC had been achieved, these survival assays were performed to investigate whether AC depleted cells displayed altered radioresistance.

The results indicated that following AC depletion with siRNA in these cell lines (HT 29, HCT 116 and LIM 1215) then they displayed decreased radioresistance and reduced survival. HCT 116 cells displayed 2.95-fold decrease in survival at 2Gy compared to non-targeting control, for HT 29 there was a 3.25-fold decrease with AC depletion. For LIM 1215 cells there was a 1.72-fold decrease in survival at 0.5Gy dosed irradiation. The results were statistically significant for all cell lines ($p < 0.05$ siAC *vs* non-targeting control).

The initial correlation between baseline AC expression demonstrated that higher expressing cell lines displayed increased radioresistance, but it is the targeted AC depletion that lends more weight to this observation. The experimental data generated from this work is supportive of the AC expression conferring radioresistance in cancer cells particularly with previous work in prostate cancer. It has been demonstrated that prostate cancer cells overexpress AC and this confers radioresistance, particularly in metastatic prostate cancer cells (Camacho et al., 2013) AC itself was found to be induced in prostate cancer cells following irradiation and in prostate cancer cells that were treated to over express AC, there was an observed increased in radioresistance (Cheng et al., 2013; Mahdy et al., 2009). AC has also been shown to be upregulated in acute myeloid leukaemia (AML) (Tan et al., 2016).

In addition to IR there is data to examine AC expression and chemoresistance. This has been shown in CRC cell lines, where AC inhibition sensitized CRC cells to oxaliplatin in vitro (Klobučar et al., 2018). In melanoma cells AC depletion with gene editing techniques has sensitized cells to doxorubicin and led to apoptosis (Lai et al., 2021). The correlation between AC expression and chemoresistance also becomes more applicable with recent evidence of the

role of anti-oestrogenic drug, tamoxifen being a potent AC inhibitor and "off target" effects of this on AC that could have interesting applications in a variety of oncology treatments (Clifford et al., 2020).

Pharmacological inhibition of AC has been described in the literature since the advent of Carmofur in the 1980s. However, there remains no routine targeted AC inhibitor in clinical practice. Various small molecular inhibitors such as LCL521 have been identified with promising *in vitro* data to show that AC can be targeted in vitro and can also act synergistically with radiotherapy and tamoxifen to produce anti-tumour effects (Bai et al., 2017). PARP inhibitors (e.g Olaparib) have been used routinely in clinical use since 2014 for breast, ovarian, prostate, lung and head and neck cancers. They were the first class of drug to be used to target the DNA damage response in BRCA1/2 breast and ovarian cancers and their exact mechanism of action is still not fully described but the main evidence supports impaired tumour cell abilities to repair DNA damage and have synergistic anti-tumour effects with radiotherapy (Fu *et al.*, 2020), these findings are similar to those observed with AC inhibition *in vitro*.

AC inhibition may have the same downstream effects as PARP inhibition or even a potential role of synergistic mechanisms with targeted inhibition of both pathways as a combined treatment.

The clonogenic data from this study serves to highlight the possibilities of AC being a novel potential target to improve response to neoadjuvant treatment and further work with pharmacological inhibition is the next logical step.

6.6 Potential mechanism underlying AC expression and resistance to apoptosis

PARP-1 cleavage immunoblotting assays confirmed that apoptosis is a key mechanism in AC depletion causing increased radiosensitivity and ultimately cell death. In HCT 116 cells they

avidly underwent apoptosis within 6h post irradiation following AC depletion (32-fold increase; $p < 0.01$). In HT 29 cells there was higher levels of apoptosis (as described by cleaved PARP-1) post irradiation in the AC depleted cells at 6h (1.7-fold increase; $p<0.05$) but higher levels at 24h (5.4-fold increase; $p < 0.3$*).

Mitochondria play a key role in cell death with cellular damage triggering membrane permeability within the mitochondria and release of pro-apoptotic cytochrome c (Cyt c) than can in turn activate the caspase pathway (Jiang and Wang, 2004). The proteins such as Bid, Bax, Bak or BH3 proteins allow pores to form that facilitate the release of pro-apoptotic proteins from within the mitochondria and there is a complex interplay with other members of the BCl-2 family which can be both pro or anti-apoptotic (Wang and Youle, 2009). More recent evidence has also highlighted the complex interplay of the endoplasmic reticulum and mitochondrial membrane mechanics in the context of apoptosis (Carter et al., 2022).

The relevance of mitochondrial instability and Cyt c release in apoptosis is important as PARP itself is a key enzyme in DNA damage response however during apoptosis it is a key substrate of caspase activity to produce specific fragments. The role of AC within this upstream pathway leading ultimately to apoptosis and PARP cleavage remains a key unanswered question from this work undertaken and the role of AC in terms of mitochondrial instability and Cyt c release could be of further interest.

Apoptosis is conventionally p53 dependent where various cellular insults, including DNA damage, cause p53 to become activated through its decoupling with E3 ubiquitin ligase MDM2. This either leads to cell-cycle arrest, DNA repair and restarting the cycle, or apoptosis. This is called the canonical pathway and relies upon protein-protein interactions. Recent evidence has shown that ceramide itself can directly activate p53 in an alternative pathway (Fekry *et al.*, 2018). This ties into with established evidence that ceramide levels increase when cells undergo DNA damage and sphingolipid homeostasis plays a crucial role in cell survival responses

following chemotherapeutic or irradiation induced DNA damage (Carroll, Donaldson and Obeid, 2015) (See Figure 6.1).

Figure 6.1 ***The intracellular ceramide synthetic pathway – the "Sphingosine Rheostat".*** Ceramide itself is pro-apoptotic and it is converted into sphingosine and fatty acids by acid ceramidase (AC). Sphingosine itself is then further converted into sphingosine 1-P which is an important promoter of cell survival. (AC – acid ceramidase, Cer – ceramide synthetase, SK – sphingosine kinase, SP – sphingosine-1-phosphatase).

The data from this experiment, particularly with the PARP-1 fragment analysis confirms higher levels of apoptosis in the cells which were AC depleted following irradiation, therefore that through AC depletion the "sphingosine rheostat" is shifted to the left through increased ceramide production and cell death. Although, ceramide levels and S1-P levels were not specifically assayed in this work the data could suggest this is occurring within the CRC lines studied and would be in keeping with literature evidence of AC modulation and apoptosis (Govindarajah et al., 2019; Lai et al., 2021).

HT 29 cells possess a mutant form of p53, although there is conflicting evidence with regards to the activity of this mutated p53. This work utilising HT 29 cells display enhanced radiosensitization to AC depletion in keeping with the other cells. The exact reason why this has been observed remains unclear; it could be due to increased ceramide levels being able to bind and activate the mutant p53 in an atypical manner with the associated downstream effects

on apoptosis or it could be to upregulation of alternative proteins such as Tap73 which can step in to mediate apoptosis in the absence of p53 (Dabiri *et al.*, 2017).

Prostate cancer cells and central nervous system tumours have also demonstrated that they can up-regulate AC expression in response to radiation as a self-selected tumour response to promote cell survival through shifting the drive towards anti-apoptotic sphingolipid metabolites (Cheng *et al.*, 2013) (Doan *et al.*, 2017).

It remains a possibility in these cell lines studied that higher expressing cell lines have evolved to over-express AC as a defence response to radiation induced DNA damage and promote cell survival hence AC depletion causing radiosensitivity and cell death.

6.7 Limitations of study

It is important to note that this work was subject to several limitations that would need to be addressed to take this body of evidence further. Firstly, suspension-based cell lines (NCI H716, NCI H508) and GEO lines are also suitable for clonogenic assays it would have employed modified techniques and within the constraints for this project, standardised clonogenic protocols were developed that were tailored towards the adherent cell lines. In the future, these suspension cell lines could be investigated separately with modified clonogenic techniques to identify whether they support the adherent cell line clonogenic data.

MDST8 cell lines were not suitable for AC siRNA clonogenic assays as once they had undergone depletion of AC they were not viable. This is an important finding as it implies that AC plays a key role within this cell line for growth and survival, further work could be undertaken with cell growth assays to investigate exactly what effects AC depletion has on this cell line irrespective of exogenous radiation therapy.

Biological inhibition of AC was used with siRNA knockdown; however these would have caused a transient AC depletion which is not a fully stable knockdown. Therefore, for a true

permanent knockdown, Clustered Regularly Interspaced Short Palindromic Repeats "Crispr Cas-9" gene editing technique could be employed however as described this technique would not be suitable for the MDST8 lines as the data suggests that AC depletion leads to cell death therefore a stable and viable clonal line would likely not be feasible. This technique could be utilised for the other cell lines in the future as they have demonstrated viability with AC depletion.

All experiments were on 2D models, ultimately 3D models and organoids could form a more realistic re-capitulation of *in vivo* tumour models for which the role of AC in radioresistance could be studied. Organoid models will display central areas of hypoxia which is a key factor in the cellular response to IR.

PARP-1 cleavage assays pointed strongly towards apoptosis as a key mechanism but there potentially remains an elegant mechanism to solidify the role of AC in promoting apoptosis in CRC cell lines and further work is needed.

HT 29 cells are p53 mutant therefore it could be argued that as apoptosis is typically a p53 dependant process, then these cells could have been compared to p53 -/- knockout models as a more valid comparator. The role of p53 in triggering apoptosis in AC depleted cells in response to IR requires further investigation.

6.8 Future perspectives

This work has helped to shape and revalidate the previous findings within this group that AC expression is implicated in a poorer response to neoadjuvant radiotherapy. However, it is the hope that this study provides a platform from which further work can be undertaken. There are several broad areas of work that could lead on from this study.

AC inhibition using the drug Carmofur is one potential option, as this drug has been used as a

5-FU derivative with potent anti AC activity in patients with breast and colorectal cancer in Japan for since the 1980s. There is also a role for small molecular inhibitors (SMIs) that are already in use in the pre-clinical stage with promising effects such as LCL 521 and the novel off target effects of tamoxifen on AC expression remain an area of future interest. The role of AC and PARP inhibition remains another potential area of pharmacological interest.

Gene editing technology (Crispr Cas-9) to create permanent AC knockout clones for suitable CRC lines and assess their survival response to radiation therapy. These techniques are

challenging to translate into 3D models therefore the need to develop 3D/organoid modelling which could then be treated with AC drug inhibitors would be of more interest.

It would be important to provide detailed mechanistic work to described which part of the "sphingosine rheostat" is manipulated through AC inhibition in CRC cells, and how that translates into controlling radiosensitivity. Pro-apoptotic Bcl-2 family members Bid and Bad are known to be important in ceramide signalling pathways (Woodcock, 2006) during cell stress and the role of AC in the upstream pathways leading to PARP cleavage remain an important area for future consideration.

Lastly, the next step would be for the development of mouse models based on these in vitro studies, as these would provide an *in vivo* model to test in principle AC as a cellular target of radiosensitization and for targeted inhibition. If demonstrated successfully, phased human trials could remain a possibility in the future.

6.9 Final conclusions

To conclude, this work serves to highlight the importance of sphingolipid metabolism in colorectal cancer and the role that AC may play in mediating the response of rectal cancer to radiotherapy. Furthermore, advanced pre-clinical models are needed to confirm these early *in vitro* studies yet there is a wide scope of potential applications for targeting AC to improve outcomes in a disease that carries such significant mortality and morbidity for thousands of patients.

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