An observational pilot study to assess the potential of a microfluidic tissue culture model to predict rectal cancer response to neo-adjuvant therapy

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Abstract

Radiotherapy has been reported to induce apoptosis and prevent the proliferation of malignant cells. Complete clinical response to neo-adjuvant long course chemoradiotherapy has been identified in up to 30% of patients with locally advanced rectal cancer. The aim of this study was firstly to maintain rectal cancer biopsies in a viable state within a microfluidic device and subsequently interrogate this *ex vivo* rectal cancer tissue with radiation and measure changes in morphology and induction of cell death through apoptosis.

Murine colorectal tissue was used for initial optimisation, followed by biopsies from patients with locally advanced rectal cancer taken prior to neo-adjuvant therapy. This tissue was maintained in a biomimetic environment within a bespoke, glass microfluidic device. Subsequently, murine tissue was interrogated with single fractions of radiation (2Gy, 10Gy or 30Gy) to identify suitable doses for delivery to human tissue. Morphology was assessed using H&E staining of the tissue. Effluent from the tissue was collected for subsequent analysis of cell death using a lactate dehydrogenase (LDH) assay and metabolite release using a mass spectrometry-metabolomics approach. Apoptosis was evaluated using the M30 CytoDeath[™] monoclonal antibody and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay to identify DNA fragmentation.

Tissue was successfully maintained for over 70 hours with evidence of viability, as determined by preservation of morphology and increased LDH release after lysis. Rectal cancer biopsies (n = 11 patients) were subsequently interrogated with radiation. Only high doses of radiation (30Gy) delivered to murine colorectal tissue reproducibly induced high levels of LDH release, however architectural losses were seen in all tissue after irradiation regardless of dose. Human tissue was therefore irradiated with 2Gy as an approximation of the dose delivered clinically.

Levels of apoptosis using M30 CytoDeathTM ELISA were not significantly increased in the irradiated groups when compared to control groups. However, using immunohistochemical assessment with M30 CytoDeathTM and TUNEL, significant increases in the irradiated groups were seen (p < 0.05). Evaluation of individual patients using these markers identified several patients with significant rises (p < 0.05) in levels of apoptosis, however there was no correlation with clinical response. Metabolomic analysis identified 28 differentially expressed (p < 0.0001) compounds in effluents collected prior to and after irradiation, however this appeared to be a time-dependent effect, rather than due to irradiation.

This work has demonstrated that the microfluidic device can be used to reliably maintain both *ex vivo* healthy murine colorectal and human rectal cancer tissue for a sufficient period of time to permit interrogation with radiation. Findings demonstrated that apoptosis and morphological changes are induced by irradiation. Further work is required to correlate findings with clinical outcome, but important progress has been made to allow use of this platform as a predictive tool of response to neo-adjuvant therapy to deliver personalised therapy.

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Glossary

3, 3'-diaminobenzidine	DAB
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
4',6-diamidino-2-phenylindole	DAPI
5-Fluorouracil	5-FU
Abdomino-perineal excision of rectum	APER
Absorbance	А
Apoptosis activating factor 1	APAF 1
Adenomatous polyposis coli	APC
American Joint Committee on Cancer	AJCC
The Association of Coloproctology of Great Britain & Ireland	ACPGBI
Bcl-2-associated X protein	bax
Bromodeoxyuridine	BrdU
Carcinoembryonic antigen	CEA
Carcinoma <i>in-situ</i>	Cis
Circumferential resection margin	CRM
Computed Tomography	СТ
Complete pathological response	pCR
Cytological grading	С
Deleted in colorectal carcinoma	DCC
Deoxyribonuclease	DNase
Deoxythymidine monophosphate	dTMP
Deoxyuridine monophosphate	dUMP
Deoxyuridine triphosphate	dUTP
Dulbecco's Modified Eagle Medium	DMEM
Endoscopic ultrasound	EUS
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Enzyme linked immunosorbent assay	ELISA
Ethylene Tetrafluoroethylene	ETFE

Familial adenomatous polyposis	FAP
Fluorescein isothiocyanate	FITC
Fraction(s)	#
Granzyme A associated DNase	GAAD
Glycogen synthase kinase	GSK
Gray	Gy
Guanine	G
Guanosine 5'-diphosphate	GDP
Guanosine 5'-triphosphate	GTP
Haematoxylin & Eosin	H & E
Head & Neck Squamous Cell Carcinoma	HNSCC
High Performance Liquid Chromatography	HPLC
Hull & East Yorkshire	HEY
Hydrochloric acid	HCl
International Union against Cancer	UICC
Lactate dehydrogenase	LDH
Liquid chromatography-mass spectrometry	LC-MS
Long course chemoradiotherapy	LCCRT
Mass-to-charge ratio	m/z
Metastases stage	M stage
Magnetic Resonance Imaging	MRI
Medical Research Council	MRC
Megavolts	MV
Minimal essential medium	MEM
Mitochondrial outer membrane permeabilisation	MOMP
Monitor Units	MU
Mouse double minute 2	mdm2
National Cancer Institute	NCI
National Institute for Health and Clinical Excellence	NICE
Nodal stage	N stage
National Cancer Institute of Canada	NCIC
Optimal cutting temperature	ОСТ

Orthogonal acceleration-time of flight	oaTOF
Phosphate buffered saline	PBS
Point of care	POC
Polydimethylsiloxane	PDMS
Polymerase chain reaction	PCR
Polyvinylidene fluoride	PVDF
Prostate specific antigen	PSA
Quality control	QC
Radio-sensitivity index	RSI
Receiver operating characteristic	ROC
Research & Development	R&D
Research Ethics Committee	REC
Short course radiotherapy	SCRT
Short interfering RNA	siRNA
Squamous cell carcinoma	SCC
Stress induced premature senescence	SIPS
Survival fraction at 2 Gray	SF2
T-cell factor	TCF
Terminal deoxynucleotidyl transferase	TdT
Terminal deoxynucleotidyl transferase (dUTP) nick-end labelling	TUNEL
Thymidylate synthase	TS
Thymine	Т
Total mesorectal excision	TME
Treatment Planning Software	TPS
Tris-buffered saline	TBS
Tumour <i>in-situ</i>	T <i>is</i>
Tumour necrosis factor	TNF
Tumour regression grade	TRG
Tumour stage	T stage
Volume by volume	v/v
Weight by volume	w/v

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Apoptosis in rectal cancer biopsies following irradiation in a microfluidic device: a predictor of response?

Oral presentation (presenting author Rikesh Patel) Society of Academic & Research Surgery Annual Meeting January 2014, Cambridge

The use of apoptotic markers to predict rectal cancer response to neoadjuvant therapy

Poster presentation (presenting author Rikesh Patel) 9th National Cancer Research Institute Cancer Conference November 2013, Liverpool

The role of microfluidic technology in response prediction of rectal cancer to neo-adjuvant therapy

Poster presentation (presenting author Rikesh Patel) European Society of Coloproctology 8th Scientific & Annual Meeting September 2013, Belgrade, Serbia

Prediction of response of rectal cancer to neo-adjuvant therapy

Poster of Distinction presentation (Awarded Certificate of Commendation) (presenting author Rikesh Patel) Association of Surgeons of Great Britain and Ireland's 2013 International Surgical Congress May 2013, Glasgow

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Graduate School Conference Presentation Fund

University of Hull *(Awarded to Rikesh Patel)* November 2013

Student Research Conference Bursary

Hull York Medical School (Awarded to Rikesh Patel) November 2013

Certificate of Commendation for Poster of Distinction

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Bowel Diseases Research Foundation (BDRF) Research Grant

A new technique to enable doctors to predict accurately how a rectal cancer will respond to radiotherapy, and so reduce the under- and over-treatment of patients (Awarded to Iain Andrew Hunter and Professor John Greenman)

December 2011

Published abstracts

Apoptosis in rectal cancer biopsies following irradiation in a microfluidic device: a predictor of response? R K Patel, V L Green, I A Hunter & J Greenman Br J Surg 2014; 101 (S4): 55 036

The use of apoptotic markers to predict rectal cancer response to neoadjuvant therapy

Rikesh Patel, Victoria Green, Rishi Srivastava, Ramsah Cheah, Iain Andrew Hunter, John Greenman NCRI Cancer Conference; 3-6 November; The BT Convention Centre, Liverpool, UK. National Cancer Research Institute (NCRI) Cancer Conference 2013; November 2013 A207 Available from: http://conference.ncri.org.uk/abstracts/2013/abstracts/A207.htm

The role of microfluidic technology in response prediction of rectal cancer to neo-adjuvant therapy

R Patel, V Green, IA Hunter & J Greenman Colorectal disease 2013; 15(Suppl. 3): 95 P223

Prediction of response of rectal cancer to neo-adjuvant therapy

R K Patel, V L Green, I A Hunter & J Greenman Br J Surg 2013; 100(Suppl. 7):99 0327

Microfluidic analysis of rectal cancer response to neo-adjuvant therapy R Patel, I.A. Hunter & J. Greenman. *Colorectal Disease 2012; 14(Suppl. 2) P226* I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material, which to a substantial extent has been accepted for the award of Doctor of Medicine (MD) in Medical sciences degree at the University of Hull, University of York or Hull York Medical School.

Signed

Printed Rikesh Kumar Patel

Date 3 March 2016

Section 1: Introduction

1.1 Background

1.1.1 Incidence and mortality

Bowel cancer was the fourth most common cancer diagnosed in the UK in 2013, with 41,112 new cases identified.(1) Cancers of the rectum and rectosigmoid junction constituted over a third of these cases (14,287; 8,958 men and 5,329 women).(1) Bowel cancer is also a significant cause of mortality and has an age standardised one-year relative survival rate in England and Wales across both sexes of 75.7%, which is reduced to 58.7% at five years (2010-2011).(1)

1.1.2 Anatomy

Anatomically the rectum is defined as the part of large bowel distal to the sigmoid colon, with its upper limit being defined as the level of the distal point of the sigmoid mesocolon (Figure 1).(2) However the Association of Coloproctology of Great Britain & Ireland Expert Advisory Committee classify a rectal tumour as any tumour whose distal margin is seen at 15 cm or less from the anal verge using a rigid sigmoidoscope.(3) Of all the cancers occurring within the large bowel (colon), it is estimated that 27% of cases are found to occur within the rectum and a further 7% at the rectosigmoid junction.(4)



Figure 1) Diagrammatic representation of the large bowel (colon)

1.1.3 Variation in incidence by ethnicity

An evaluation of bowel cancer registration data across England between 1996 and 2004 analysed by Cancer Research UK, demonstrated significantly lower rates of bowel cancer incidence amongst both Asian and Black populations (Table 1).(5) However, the reasons for this are not yet understood.

Ethnicity	White		Asian		Black	
Sex	Male	Female	Male	Female	Male	Female
Age-standardised rate	54.1-	34.0-	19.1-	11.3-	29.7-	20.4-
per 100,000	55.3	34.8	28.0	17.5	43.8	31.6

Table 1) Adapted from Cancer research UK "Bowel cancer incidence byethnicity"(5)

1.2 Disease progression

The adenoma-carcinoma sequence has been acknowledged to be a common process by which normal, healthy colorectal tissue evolves to become neoplastic.(6) Although there is an abundance of epidemiological, clinical, histopathological and genetic data supporting this, there is still a lack of conclusive evidence.(7–9) As with other tissue, epithelial cells of the colorectal tract are continuously replaced, however genetic mutations in this reparative process can lead to progression of healthy epithelial tissue to form pre-malignant adenomas, which then progress to carcinoma *in situ* (C*is*) and then finally to invasive carcinomas. In addition to this adenomacarcinoma sequence, another proposed mechanism is the development of cancers *de novo*.(10–12)

1.2.1 Epidemiology

Examination of age distribution curves demonstrate an increasing prevalence of both adenomas and carcinomas with age, but the prevalence of adenomas is shown to peak between five to ten years prior to that of carcinomas.(13,14) Clark et al performed a multi-centre autopsy study to examine the relationship between the prevalence of adenomas and the incidence of colorectal carcinomas.(15) The areas of the colon with the highest proportion of large adenomas were the caecum/ascending colon (36%) and the sigmoid colon (25%). These regions were also found to have high incidences of cancer; caecum/ascending colon (20%) and sigmoid colon (23%). Despite the rectum containing the lowest proportion of adenomas (8%), the highest proportion of colorectal cancers (39%) was identified. Unfortunately no statistical analysis was performed to identify if this was significant. In addition, despite adenomas being identified in 30-40% of people by the age of 60, the lifetime cumulative incidence of colorectal cancer is only 5.5%. This would suggest that not all adenomas progress to become malignant: a finding confirmed by several authors.(13,16,17)

Clark pointed out that these findings do have several possible implications

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with respect to rectal cancer: either a greater proportion of rectal in comparison to colonic adenomas progress to invasive cancer, which was supported by the finding of rectal adenomas being of a greater diameter than those within other colonic segments; or the adenoma-carcinoma sequence appears to play less of a role in the development of rectal cancer.

1.2.2 Clinico-pathology

Progression of colorectal polyps left *in-situ* has been followed by studies performed before the advent of colonoscopic removal of polyps (polypectomy). Hoff *et al* followed up 215 colorectal polyps of less than 5mm that were left *in-situ* over a two-year period.(18) Although growth and regression of the polyps was identified, none of the cases demonstrated evidence of severe dysplasia or carcinoma over this period. Stryker *et al* performed a retrospective review over a six year period (mean follow-up of 68 months), where polyps greater than or equal to 10mm were followed up radiologically.(19) Over the follow-up period, 37% (83/227) of polyps enlarged and 21 invasive carcinomas were identified at the site of the index polyp. This would indicate that the timeframe of follow-up is critical. Upon histopathological examination of resected polyps, malignant foci have been identified within presumed adenomas, with between 6.4% and 12.4% containing Cis and between 2.7% and 5.3% containing invasive carcinoma.(16,20–22) Colorectal carcinomas have also been shown to contain elements of benign adenomatous tissue. In Muto *et al*'s series of 1961 malignant tumours, 261 (14.2%) had evidence of contiguous benign tumour.(13) This finding has been replicated by several other studies: Eide et al (23%)(23), Adachi et al (17%)(24) and Bedenne et al (15.5%)(10). Endoscopic polypectomy has been shown to reduce the risk of development of colorectal cancer.(25–27) Guidelines based on these findings have been created in order that surveillance colonoscopy is performed.(17)

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1.2.3 Molecular Genetics

1.2.3.1 Adenomatous Polyposis Coli (APC) gene

Germline mutations of the APC tumour suppressor gene on chromosome 5q21, have been shown to predispose to familial adenomatous polyposis (FAP) coli, which is an autosomal dominant disorder that leads to the development of multiple adenomatous colonic polyps by early adulthood.(28) These polyps, if left untreated progress to colorectal cancer by an average age of 35 to 40 years. A loss of function of the gene encoding for APC has been identified in up to 85% of all cases of colorectal cancer.(29) The gene encodes for a 312kDa protein, which is multifunctional and has multiple domains able to interact with several other proteins including βcatenin, glycogen synthase kinase (GSK) 3β and end binding protein.(30) The normal function of the APC protein is to form a destruction complex by binding with GSK 3 β , β catenin and axin, which leads to proteolytic degradation. In the presence of the Wnt signal, β -catenin cannot be bound and hence accumulates within cells. β -catenin is then free to bind to the Tcell factor (TCF) family of transcription factors and activate gene transcription (Figure 2). In the presence of an APC gene mutation, this regulation of β -catenin is lost and subsequently leads to increased transcriptional activity for proliferation genes and has been identified in colorectal tumours.(31)



Figure 2) Simplified diagram of Wnt pathway and regulation of β -catenin adapted from Moon *et al.*(32) **A)** Formation of the destruction complex leads to proteolysis of β -catenin. **B)** Wnt binds to the receptor and removes axin from the destruction complex and stabilised β -catenin moves into the nucleus to activate protein transcription. **C)** In the presence of mutant APC, a destruction complex cannot be formed leading to accumulation of stabilised β -catenin, which is able to initiate protein transcription, regardless of Wnt binding. This leads to the uncontrolled cell growth seen in cancer.

(B-cat – β-catenin; GSK – GSK 3β; mAPC – mutant APC; TCF – T-cell factor)

A comparable incidence of mutations of the APC gene or sequences on chromosome 5 that have been linked to FAP have been identified in both adenomas and colorectal cancers.(9) However, despite these mutations occurring at a similar incidence at all stages, Miyaki *et al* identified an increased loss of heterozygosity from the progression of moderate adenoma (1%) to severe adenoma (15%) to intra-mucosal carcinoma (26%) to invasive carcinoma (44%).(33) This trend has been observed in both FAP and non-FAP patients. In patients with colorectal tumours, where APC mutations were not identified, β -catenin mutations have been observed.(34,35)

1.2.3.2 K-ras (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)

The ras family of proteins are responsible for cellular signal transduction pathways, which are important in ensuring normal proliferation and differentiation.(36) A loss of function of the gene encoding for K-ras has been identified in 50-60% of all cases of colorectal cancer.(29) The K-ras oncogene encodes for a 21kDa protein that is activated by the binding of ligands to the epidermal growth factor receptor (EGFR), which sets off a cascade of events that induces gene transcription by G protein signalling. The active K-ras protein is bound to guanosine 5'-triphosphate (GTP), but hydrolyses this to form guanosine 5'-diphosphate (GDP) (Figure 3).(37)



Figure 3) Simplified diagram of K-ras pathway demonstrating K-ras inducing gene transcription when in its active state adapted from Walther *et al.*(38) Dimerisation of the epidermal growth factor receptor (EGFR) occurs upon ligand binding, leading to intra-cellular kinase domain activation. The K-ras cascade is activated via small adaptor proteins, Sos and Grb and subsequently recruit BRAF. In turn MEK and ERK kinases are triggered through phosphorylation to promote cell proliferation and survival.

These mutations decrease the ability of K-ras to hydrolyse GTP and hence it continues to remain in its active state to continually induce cell growth, proliferation and survival. Mutations in several hot spots of the K-ras oncogene are present in 35-43% of colorectal cancers.(39–41) K-ras mutations have also been identified in in 27-65% of adenomas greater than 1cm in size, but this prevalence is lower in adenomas smaller than this, which suggests that the role of K-ras does not occur until after the adenoma has already become established.(40,42,43) Morris *et al* identified a statistically significant difference in incidence of Kras mutations found in large (>10 mm) dysplastic adenomas in patients with or without concurrent colorectal carcinomas; 48.4% (15/31) vs. 23.1% (12/31) respectively (p = 0.028).(44) However, when looking specifically at dysplastic adenomas in the rectum and sigmoid colon, this was only found to be significantly higher in patients over the age of 70 and only twenty-six adenomas were analysed in this subgroup (p = 0.026). In tissue resected from patients with colorectal cancers found to have K-ras mutations, histologically normal tissue was also found to be positive in up to 53.8%.(45,46) Zauber *et al* found dysplastic adenomas and the malignant sections of carcinomas*-in-situ* are significantly more likely to contain K-ras mutations than colorectal carcinomas, 61.9%, 67.8% and 31.6% respectively (p < 0.0001).(47) This would suggest that K-ras may not play as great a role in late tumours with a possible loss of the mutation during progression of disease.

Anti-EGFR drugs, such as cetuximab, a monoclonal antibody have been used in the treatment of advanced metastatic colorectal cancer, in addition to other chemotherapeutic drugs.(48) Their mechanism of action is to bind to EGFR an thus prevent uncontrolled growth and as a result K-ras mutations have been associated with a worse prognosis.(49,50) Cetuximab is therefore only used patients without K-ras mutations and mutation status is assessed prior to commencement.

1.2.3.3 p53

The p53 gene, frequently referred to as 'the guardian of the genome', is a tumour suppressor gene, which has been mapped to chromosome 17p and has been identified to be mutated in over 75% of colorectal carcinomas.(51) This would suggest that impaired p53 is advantageous for tumour cells. p53 acts as a transcription factor that binds to specific DNA sequences and transactivates genes involved in a variety of functions including apoptosis, senescence and arresting the cell cycle to allow DNA repair regulation (Figure 4).(52–54)

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Figure 4) A) An overview of p53 function within a normal cell, demonstrating that in the presence of DNA damage or other insults, p53 is dissociated from mdm2, a negative regulator and is activated. This results in cell cycle arrest to permit repair or initiate apoptosis. **B)** When p53 is mutated, cell cycle arrest is not induced and as a consequence DNA repair cannot occur and cells do not undergo apoptosis. This results in further replication and potential amplification of genomic instabilities. Adapted from Sengupta and Harris.(55) (BAX – bcl-2-associated X protein, mdm2 – mouse double minute 2)

However, mutant p53 present in cancer cells is unable to bind to DNA to regulate cell proliferation, resulting in incomplete DNA repair and mutated cells. The half-life of mutant p53 has been shown to be prolonged when compared to that of wild-type p53 (1-24 hours and 6-20 minutes respectively).(56) p53 mutations have been estimated to account for greater than 50% of human cancers.(52)

1.2.3.4 Loss of 18q heterozygosity

An increase in the loss of heterozygosity of 18q has been identified with progression of colorectal adenomas to carcinomas and across progressive stages of cancer.(57–59) Vogelstein *et al* identified an increase in the loss of heterozygosity in allele 18q from up to 13% in adenomas to 47% in advanced adenomas and 73% in carcinomas.(40) Deleted in Colorectal Carcinoma (DCC), a tumour suppressor gene has been identified on chromosome 18q.(60)

1.2.3.5 Methylation status

Post-translational modifications occur on proteins catalysed by enzymes after completion of translation by ribosomes and involves the covalent addition of a functional group.(61) Methyl groups are added to DNA to control gene expression and are essential for development and normal functioning. Errors in methylation have been identified to lead to a variety of diseases with cancerous cells identified to be both hypo- and hypermethylated in relation to normal adjacent cells.(62,63) Hypomethylation has been observed in both colonic benign and malignant lesions.(64,65) Hypermethylation of the APC promoter region has also been observed in cases of colorectal cancer in relation to healthy mucosa. Although Esteller *et al* reported hypermethylation in relation to surrounding healthy colorectal tissue of the APC promoter region present in 18% (20/108) of primary colorectal carcinomas and also in 18% (9/48) of both small (<15mm) and large (>15mm) adenomas, Hiltunen *et al* identified only an increase in actual malignancies and not in pre-malignant lesions.(66,67)

1.2.4 Cytogenetics

Tsafrir *et al*'s study identified changes in expression with both over- and under-expression occurring in several groups of contiguous genes with disease progression from normal tissue to adenoma to carcinoma and to metastatic disease(68) Over-expression was seen in chromosomes 7, 8q, 13 and 20, whereas under-expression was found in 1p, 4, 5q, 8p, 14q, 15q, 17p and 18. This supported the previous findings of several other authors who noted more marked chromosomal abnormalities with progressive disease.(69–71)

1.3 Diagnosis of rectal cancer

If there is a concern regarding the possibility of a colorectal malignancy and the patient's General Practitioner (GP) feels that investigation is warranted, a 'fast track' referral can be made to a Colorectal unit, using the "Referral guidelines for suspected cancer – Clinical Guideline 27" developed by the National Institute for Health and Clinical Excellence (NICE) (June 2005).(72) This allows patients with high-risk symptoms and signs to be assessed within two weeks of referral (Table 2).

Age	Symptoms/Signs	Length of
		time
40 years and	Rectal bleeding with a change of bowel habit towards	6 weeks or
older	looser stools and/or increased stool frequency	more
60 years and	Rectal bleeding without a change in bowel habit and	6 weeks or
older	without anal symptoms	more
60 years and	Change in bowel habit to looser stools and/or more	6 weeks or
older	frequent stools without rectal bleeding	more
Irrespective	Right lower abdominal mass consistent with	N/A
of age	involvement of the large bowel	
Irrespective	Palpable rectal mass (intraluminal and not pelvic)	N/A
of age		
Irrespective	Unexplained iron deficiency anaemia and a	N/A
of age	haemoglobin of 11g/dl or below (Male) or 10g/dl or	
	below (Female)	

Table 2) A summary of NICE guidelines suggesting prompt referral to rule out colorectal cancer.

1.3.1 Clinical findings

Common symptoms of a left sided (sigmoid or rectal) colorectal cancer include rectal bleeding and a change of bowel habit, most commonly with an increased frequency and/or looser stools. Patients presenting with symptoms of rectal bleeding without associated anal symptoms, should be referred as part of two week standard in order that rectal cancer can be excluded.

GPs should perform a digital rectal examination as part of their abdominal examination, as a palpable mass may be identified and thus prompt urgent investigation.

1.3.2 Investigations

Investigations are dependent on the presenting features. Patients that present with rectal bleeding and/or a change in bowel habit with no other significant associated factors, that are found to have a malignancy, tend to occur in the left side of the bowel (within 60cm of the anal verge). Diagnosis can be obtained via flexible sigmoidoscopy, which is performed at the twoweek wait clinic. If a rectal malignancy is identified at this clinic, the patient will require a visualisation of the remainder of the colon, as synchronous colorectal cancers have been identified in up to 5% of patients. The colon can be fully visualised either using colonoscopy, double contrast barium enema, in addition to endoscopic visualisation of the rectum (flexible sigmoidoscopy), or CT colonography. However it is worth noting that only colonoscopy out of these methods can provide direct visualisation of the bowel wall and thus also perform biopsies of potential areas of abnormality. Colonoscopy has been shown to be superior in diagnosing colorectal malignancies, due to its higher sensitivity, with rates of 79-100%.(73–75) However, despite this increased sensitivity, colonoscopy is an invasive procedure and therefore should be reserved for high-risk patients due to its higher rate of complications, such as iatrogenic perforation, which may require operative intervention in up to 0.196%.(76–79)

1.4 Pre-operative investigations in rectal cancer

If there is high suspicion of a rectal malignancy on endoscopy and histological biopsies have been taken, it is important that further preoperative investigations are carried out in a timely manner to further classify the cancer and ensure that treatment is commenced within 62 days of the initial referral.(80) This also allows decisions to be made with regards to potential treatment options.

1.4.1 Factors determined by investigations

The staging investigations ascertain the local extent of the disease, lymph node involvement and metastatic spread. High resolution MRI in combination with CT are the recommended investigations by the Royal College of Radiologists.(81) These are used to determine the following factors:

- Potentially irresectable disease
 - If the tumour is not resectable surgically, it may benefit from chemoradiotherapy to downstage and shrink the tumour as discussed in section 1.7. However, if the extent of disease is so significant, resectional surgery may not be felt to be beneficial and thus would likely be managed palliatively.
- Length of tumour and location in relation to the anal verge
 - This information permits a decision to be made as to the type of surgery offered as described in section 1.6.
- Degree of local spread within the mesorectum
 - In addition to leading to potentially inferior outcomes, local spread affects the decision as to whether adjuvant therapy would be beneficial. This is described in more detail in section 1.7.
- Presence of adverse features including nodal spread, extramural venous invasion and infiltration of the peritoneum
 - Again, as well as leading to potentially inferior outcomes, this invasion affects the decision as to whether adjuvant therapy would be beneficial.
- Presence of metastatic lymph nodes outside the mesorectum including external and common iliac regions

- Presence of complications including obstruction or perforation
 - These findings can lead to a worse morbidity and mortality and in addition warrant urgent surgical intervention.(82,83) The patient may need to be temporarily defunctioned with a stoma without undergoing any form of resectional surgery at that time.(84)
- Metastatic disease including lung and liver metastases.
 - In the presence of distal metastases, the decision as to whether additional surgery (e.g. liver or lung resection) would be beneficial has to be made.

Survival and clinical outcomes in rectal cancer have been shown to be significantly dependent on the following factors: depth of tumour, extramural venous invasion, circumferential resection margin positivity, regional lymph node involvement, peritoneal invasion/perforation, as well as distal metastases.(85)

1.5 Staging classifications of rectal cancer

There are several different methods of staging used throughout the world in order to stage rectal cancer. Methods currently used by the Association of Coloproctology of Great Britain & Ireland include a modified Dukes' system and the TNM system.

1.5.1 Dukes staging

Dukes first described the staging of rectal cancer in 1932 to aid prognosis, where he described three stages (Figure 5)(86):

- A) Carcinoma confined to rectal wall with no extension to surrounding tissues or lymph nodes
- B) Carcinoma that has spread directly to adjacent tissue, but no involvement of lymph nodes
- C) Metastases in regional lymph nodes



Figure 5) A diagrammatic representation of Dukes' classification adapted from Cancer Research UK.(87)

1.5.2 Modified Astler-Coller Dukes staging

An adaptation of the Dukes system was suggested by Astler and Coller in 1952.(88) Now more commonly a modified version of this is used which involves Turnbull's addition of a further stage, D in 1967 (Table 3).(89)

Table 3) Modified Astler Coller Dukes staging with Turnbull's additionalstaging of metastatic cancer.

Stage	Level of invasion of malignancy
А	Lesions limited to the mucosa
B1	Lesions extending into the muscularis propria but not penetrating it,
	without regional nodal metastasis
B2	Lesions penetrating the muscularis propria, without regional nodal
	metastasis
B3	Lesions invade adjacent structures, without regional nodal metastasis
C1	Lesions extending into the muscularis propria but not penetrating it, with
	regional nodal metastasis
C2	Lesions penetrating the muscularis propria, with regional nodal metastasis
C3	Lesions invade adjacent structures, with regional nodal metastasis
D	Extensive local spread or with distal metastases

1.5.3 TNM staging

The TNM staging system, developed by Pierre Denoix between 1943 and 1952 is now more commonly used. It is continually reviewed and updated by the International Union against Cancer (UICC) and is identical to the American Joint Committee on Cancer (AJCC) staging classification.(90,91) It describes three categories that are relevant to prognosis. The T stage describes the invasion of the primary tumour (Figure 6); the N stage describes the extent of spread to regional lymph nodes; and the M stage describes metastases, i.e. whether the tumour has spread to other organs within the body (Table 4).

Clinical stage and pathological stage are denoted by a lower case "c" or "p" respectively before stage (e.g. cT3N1M0 or pT2N0). The y prefix is used for cancers classified after neo-adjuvant treatment (e.g. ypT1N0).

Primary tumour (T)	Regional lymph nodes (N)	Distant metastasis
		(M)
T0 – No evidence of	Nx – Regional lymph nodes	M0 – No distant
primary tumour	cannot be assessed	metastasis
Tis – Tumour <i>in situ</i>	N0 – No regional lymph node	M1a – Metastasis
	metastasis	confined to one
T1 – Tumour invades	N1a – 1 regional lymph node	organ or site
submucosa	N1b – 2-3 regional lymph nodes	M1b – Metastases in
T2 – Tumour invades	N1c – Tumour deposit(s) in the	more than one
muscularis propria	subserosa, mesentery or non-	organ/site or the
T3 – Tumour invades	peritonealised pericolic or	peritoneum
through muscularis	perirectal tissue without nodal	
propria into	metastasis	
pericolorectal tissues		
T4a – Tumour	N2a – metastasis in 4-6 regional	
penetrates to the	lymph nodes	
surface of the visceral	N2b – metastasis in 4-6 regional	
peritoneum	lymph nodes	
T4b – Tumour directly		
invades or is adherent		
to other organs or		
structures		

Table 4) TNM staging adapted from UICC/AJCC Colon and Rectum CancerStaging 7th Edition(91)



Figure 6) A diagrammatic representation of T stage classification (TNM) adapted from Cancer Research UK.(92)

1.5.4 Comparison of TNM, Dukes and Modified Astler-Coller Dukes staging

A comparison of the various staging systems discussed above is shown in Table 5. It can be seen that the TNM classification provides a more detailed approach and also classifies *in-situ* neoplasms (T*is*) otherwise known as C*is*.

Table 5) UICC/AJCC Colon and Rectum Cancer Staging 7th Edition and acomparison with Dukes' and Modified Astler-Coller staging

Stage	Т	Ν	Μ	Dukes	Modified Astler-Coller
					Dukes
0	Tis	N0	M0	-	-
Ι	T1	N0	M0	А	А
	T2	N0	M0	А	B1
IIA	Т3	N0	M0	В	B2
IIB	T4a	N0	M0	В	B2
IIC	T4b	N0	M0	В	B3
IIIA	T1-T2	N1a/b/c	M0	С	C1
	T1	N2a	M0	С	C1
IIIB	T3-T4a	N1a/b/c	M0	С	C2
	T2-T3	N2a	M0	С	C1/C2
	T1-T2	N2b	M0	С	C1
IIIC	T4a	N2a	M0	С	C2
	T3-T4a	N2b	M0	С	C2
	T4b	N-1-N2	M0	С	C3
IVA	Any T	Any N	M1a		D
IVB	Any T	Any N	M1b		D

1.6 Evolution of surgical techniques in the management of rectal cancer

1.6.1 Abdomino-perineal excision of the rectum (APER)

Miles first described the role of the abdomino-perineal excision of the rectum, along with its associated lymph nodes en bloc in order to improve rectal cancer survival (Figure 7).(93) Prior to this, the majority of rectal cancer surgery was carried out through the perineum and Miles reported early recurrences in 95% of perineal resections carried out prior to this. However, the main disadvantage of this extensive resection was that patients were left with a permanent stoma, irrespective of the position of the cancer.



Figure 7) Abdomino-perineal excision of rectum: A: Due to the low position of the tumour, in order to obtain a clear margin of tissue distal to the tumour, the anus is resected along with the rectum. The procedure involves surgery through both the abdomen and the perineum. B: The patient is left with a permanent end colostomy and the perineal wound is closed. Adapted from Cancer Research UK.(94)

1.6.2 Anterior resection

Dixon later reported the results of a less radical, sphincter-saving procedure, the anterior resection, for cancers of the upper and middle third of the rectum.(95) This procedure involves removal of the part of the rectum in which the cancer lies, but allowing for adequate resection margins, the remaining ends are anastomosed to resume continuity of the bowel (Figure 8). In his series of 400 patients, mortality rates of 2.6% and five-year survival rates of 64% were reported. This led to the increased role of performing sphincter-saving procedures and avoiding stoma formation, yet offering patients an equivalent outcome.



Figure 8) Anterior resection: **A)** The tumour within the rectum is excised along with a margin of surrounding healthy tissue either side of it. **B)** An anastomosis is formed between the remaining rectum and colon. A temporary defunctioning ileostomy may be formed in order to allow the new anastomosis to heal with the aim of later reversal to resume continuity of the bowel. Adapted from Cancer Research UK.(94)

1.6.3 Total mesorectal excision

Currently total mesorectal excision (TME) is recommended by The Association of Coloproctology of Great Britain & Ireland for cancer in the lower two thirds of the rectum, either as part of a low anterior resection or an abdomino-perineal excision of rectum (APER).(3) TME involves sharp dissection in the plane that separates the visceral mesorectal fascia from the parietal pelvic fascia to remove the entire mesorectum and its associated lymph nodes. The technique was first described by Heald *et al* in 1982 who demonstrated that it was an efficient way of reducing local recurrence rate to 3% at a median follow-up of 4.2 years.(96,97)

Ouirke *et al* reviewed whole-mount sections of resected rectal adenocarcinoma specimens and identified that involvement of the circumferential resection margin was related to local pelvic recurrence.(98) In their study, 14 of their 52 (27%) patients with rectal adenocarcinoma had involvement of the circumferential resection margin. Twelve of these patients with involved margins had subsequent local recurrence. The importance of a clear circumferential margin first identified by Quirke has been reproduced in several studies, where local recurrence was found to be significantly higher in those patients with involved margins.(99–101) Heald reinforced the role of TME, where he operated on 519 patients with rectal adenocarcinoma over a 19-year period.(102) In this relatively large case series, local recurrence rate was shown to be 6% at five years and 8% at ten years after surgery. In patients who had undergone a curative resection, the rate of local recurrence was 3% over five years and 4% at ten years, as well as having disease free survival rates of 80% at five years and 78% at ten years. Similar local recurrence rates were also reported by Enker et al and Aitkin, who reported rates of 7.3% at five years. (103) Subsequently, Arbman *et al* compared the outcomes between two cohorts undergoing curative surgery for rectal cancer before and after the introduction of TME. Local recurrence at one year had developed in 19/134 (14%) patients prior to TME and in 8/128 (6%) after the introduction TME (p = 0.03). However, it is worth noting that only 67/128 (52%) patients in the second group

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underwent TME. This finding has been confirmed by several studies, where improved outcomes have been attributed to the use of TME.(104–106) There is also a recommendation by the Association of Coloproctology of Great Britain & Ireland that local excision for cure in rectal cancers should only be performed for T1 tumours (i.e. tumours that are less than 3cm diameter and are well to moderately differentiated).(3)

1.7 Locally advanced rectal cancer

A proportion of rectal cancers can be managed by surgery alone, using either local resection or TME, with a relatively low risk of local recurrence, whereas there are also patients in whom TME will result in a clear circumferential resection margin (CRM), but will develop local recurrences.(107) Therefore in these patients where local recurrence is a concern, neo-adjuvant therapy may be recommended. Locally advanced rectal cancer has been defined as being "fixed on palpation or involving or threatening the CRM on MRI".(3) This has commonly been classified using the TNM classification as T3 or T4 and/or N1 or UICC/AJCC classification stage III.(108,109)

1.8 Adjuvant therapy

Adjuvant treatment has been demonstrated in several studies to be beneficial in the management of rectal cancer.

1.8.1 Pre-operative versus post-operative therapy

It has been shown that chemoradiation given pre-operatively (neoadjuvant) instead of post-operatively results in a lower incidence of toxicity. Minsky *et al* carried out a study comparing subsets of patients from two parallel phase I trials.(110) Although the study was limited by the fact that the patient groups were not randomised; they identified severe or lifethreatening toxic events to occur in 13% of those treated pre-operatively vs. 48% in those treated post-operatively (p = 0.045). Subsequently, at the end of the twentieth century, three randomised phase III trials were initiated that compared the use of pre-operative chemoradiotherapy with that given post-operatively. Sauer *et al* published the work of the German CAO/ARO/AIO trial, where they recruited over 800 patients with locally advanced rectal cancer and randomised them to receive either long course pre-operative or post-operative chemoradiotherapy.(111,112) Severe or life-threatening side-effects according to common toxicity criteria were reported to be lower in those treated pre-operatively than those treated post-operatively; 27% vs. 40% respectively (p = 0.001). Five- and ten-year local recurrence rates were also significantly lower in those treated with pre-operative chemoradiotherapy; 6% vs. 13% (*p* = 0.006) and 7.1% vs. 10.1% (*p* = 0.048) respectively. These adverse effects were also shown to persist long-term and these again were significantly lower in the pre-operative group; 14% vs. 24% (p = 0.01). However, despite these findings, a significant difference in ten-year overall survival was not seen; 59.6% in the pre-operative group and 59.9% in the post-operative group (p = 0.85).

The other two trials, the Intergroup 0147 and the National Surgical Adjuvant Breast and Bowel Project R-03 (NSAP R-03) did not come to completion due to insufficient patient recruitment.(113) Roh *et al* published the limited results of the NSAP R-03 trial, where 267 randomised patients either underwent pre-operative or post-operative chemoradiotherapy.(114) Although the rate of local recurrence was reported to be 10.7% in each group, five-year disease free survival was significantly higher in those treated pre-operatively; 64.7% vs. 53.4% (p = 0.011). The rate of severe or life-threatening toxicity was found to be higher in the pre-operative group than the post-operative group; 33% vs. 23%; but as the study was powered with the aim of recruiting 900 patients, statistical significance was not reached.

The Medical Research Council (MRC) CR07 and the National Cancer Institute of Canada (NCIC) Clinical Trials Group C016 multicentre, randomised controlled trial evaluated the use of short-course neo-adjuvant radiotherapy
compared with the use of selective adjuvant (post-operative) chemoradiotherapy in patients with involved circumferential margins.(115) Over a period of seven years, 1350 patients with locally advanced rectal cancer were recruited across 80 centres. The patients were assigned to either short course neo-adjuvant radiotherapy consisting of 25 Grays (Gy) in five daily fractions (n = 674) or to surgery followed by selective adjuvant chemoradiotherapy consisting of 45Gy in 25 fractions with 5-FU in patients with circumferential resection margin involvement of ≤ 1 mm (n = 676). Of the patients undergoing anterior resection, no significant difference in rate of anastomotic leak was found between the two groups; neo-adjuvant therapy: 9% vs. selective adjuvant therapy: 7%. However, there was a greater incidence of non-healing perineal wounds after APER in patients who had received neo-adjuvant therapy; 35% vs. 22%, but despite this, rates of overall healing at 12 and 24 months did not differ. Of the 646 patients allocated to the selective adjuvant chemoradiotherapy group, 77 (12%) had involved circumferential resection margins. Of these 77 patients, 53 (69%) underwent chemoradiotherapy, seven (9%) underwent radiotherapy only, 15 (19%) did not undergo further treatment and the remaining two patients had missing treatment data. Pre-operative radiotherapy was proven to beneficial in terms of local recurrence (reduction in relative risk of 61%; *p* < 0.0001) and disease-free survival (relative improvement of 24%; p = 0.013) when compared to selective postoperative chemoradiotherapy, however, a difference was not demonstrated with regards to overall survival.

Given the overall evidence, NICE have advocated the use of neo-adjuvant therapy over adjuvant therapy in the treatment for locally advanced rectal cancer, due to fewer and less severe side effects and lower rates of local recurrence, in addition to disease-free survival.(116)

1.8.2 Pre-operative (neo-adjuvant) therapy

Use of pre-operative chemoradiotherapy has also been demonstrated to result in downstaging that can allow sphincter-preserving procedures and

potentially even allow local resection.(117) Pre-operative radiotherapy in the treatment of rectal cancer is most commonly performed either by conventional fractionation as long course chemoradiotherapy (LCCRT), or as short course radiotherapy (SCRT):

LCCRT consists of radiotherapy doses of 45-50Gy in 25 daily fractions over five weeks with concurrent 5-fluoracil (5-FU) based chemotherapy and surgery is scheduled four to eight weeks after completion of radiotherapy, allowing the tumour to shrink prior to resection.(118,119) SCRT is given as a dose of 25Gy over five daily fractions over a week.(118,119) The aim of SCRT is to reduce the risk of local recurrence and surgery is usually carried out within ten days of completing treatment, prior to the onset of acute adverse effects due to the radiotherapy. As SCRT does not significantly shrink the tumour, it is not appropriate for rectal cancers that are clinically or radiologically irresectable.

The Swedish Rectal Cancer Trial was the first study to demonstrate a significantly lower rate of local recurrence with the use of pre-operative SCRT followed by surgery within a week, compared to surgery alone.(120) Over a three-year period, 1168 patients with resectable rectal cancer were randomly assigned to one of these cohorts. They reported local recurrence rates of 63/553 (11%) in the cohort treated with SCRT and 150/557 (27%) in those not receiving neo-adjuvant therapy (p < 0.001) and improved overall survival (58% vs. 48% respectively; p = 0.004) at five years. Long-term results of the trial (median follow-up 13 years) revealed an overall survival rate of 38% in SCRT cohort compared to 30% in the cohort that only underwent surgery (p = 0.008). Improved cancer specific survival rates (72% vs. 62% respectively; p = 0.03) and local recurrence rates (9% vs. 26% respectively; p < 0.001) were also found in the irradiated group when compared to the non-irradiated group.(121)

The Dutch Colorectal Cancer Group's prospective, multicentre randomised controlled trial investigated the efficacy of pre-operative SCRT combination with standardised TME in patients with locally advanced cancer.(122) The rate of local recurrence at two years was 2.4% in the irradiated group vs.

8.2% in the group that underwent surgery alone (p < 0.001). However, no significant difference was found in overall survival at two years between the two groups. The ten-year cumulative incidence of local recurrence was 5% in the irradiated group compared to 11% in the group that underwent surgery alone (p < 0.0001); however, ten-year overall survival probabilities were 48% and 49% respectively (p = 0.86). When assessing cancer-specific death, the cumulative incidence was not significantly different (28% vs. 31% respectively; p = 0.20).(123)

Svoboda *et al* examined the use of 'sandwich therapy', where the use of a combination of pre-operative and post-operative radiotherapy were compared with use of only pre-operative radiotherapy.(124) The pre-operative radiotherapy consisted of four fractions of 5Gy given within two to three days, a protocol used between 1986 and 1990 at the University Hospital of Hamburg. During this time period, 146 patients received pre-operative radiotherapy, with a further 63 also receiving post-operative radiotherapy. 'Sandwich therapy' vs. pre-operative radiotherapy was shown to result in an increased frequency of severe late complications (84% vs. 17% at five years respectively) without increasing local tumour control (88% vs. 90% at five years respectively).

The use of neo-adjuvant therapy has been found to improve outcomes such as local recurrence, however there is still debate as to whether overall survival is increased. Despite this, current NICE guidelines would support the use of neo-adjuvant therapy with at least a moderate risk of local recurrence, after discussion with the patient of the risks of local recurrence and potential late toxic effects of therapy.(116)

1.8.3 Short course radiotherapy (SCRT) versus Long course chemoradiotherapy (LCCRT)

Throughout the world there is a debate over which offers the superior outcome between use of neo-adjuvant SCRT and LCCRT. Bujko *et al* performed a randomised controlled trial on behalf of the Polish Colorectal Study Group looking at 312 patients across 19 centres. (125) Patients either received SCRT followed by surgery within seven days (n = 155) or LCCRT (50.4Gy in 28 fractions) followed by surgery four to six weeks later (n = 157). Their primary endpoint was sphincter preservation, but at their median follow-up of 48 months, no statistical difference was found between the two groups; 61.2% in after SCRT and 58.0% after LCCRT (p = 0.57). There was also no difference identified in the rate and severity of postoperative complications. However, rates of overall pathological complete response were shown to be superior with LCCRT (16.1 vs. 0.7%) with positive circumferential margins also less common in this group (4.4% vs. 12.9%; p = 0.017). Despite these findings, no significant difference was seen in overall or disease-free survival at the median follow-up of 48 months. Although this study would suggest that LCCRT is not beneficial when compared to SCRT, the authors acknowledge that a limitation of their study is that as it has been powered to detect differences of 15% or more and therefore small differences are not likely to be identified. Also despite the inclusion criteria only including patients with T3/T4 disease, 39.5% of resected specimens of patients in the SCRT were found to have T1/T2 disease, which is unlikely to be due to downstaging.

Ngan *et al's* more recent randomised trial of 323 patients across 27 centres again compared the same two modalities, using local recurrence at three years as their primary outcome.(126) At three years, the cumulative incidence of local recurrence was not statistically different between the two groups; 7.55% in the SCRT group vs. 4.4% in those undergoing LCCRT (p =0.24). Again at five years, this difference was not found to be significant (SCRT 7.5% vs. LCCRT 5.7%; p = 0.51). Overall survival rates between the two groups were again not found to be statistically different (LCCRT 70% vs. SCRT 70%; p = 0.62. LCCRT in this study was associated with a greater downstaging of T stage (45% vs. 28%; p = 0.002). Despite this, the rate of APER's required for distal tumours (<5cm from the anal verge) was not altered (LCCRT, 38 of 48 (77%) patients vs. SCRT 23 of 30 (79%) patients; p= 0.87). The authors acknowledged that LCCRT may be more effective in reducing the risk of local recurrence, despite not being able to identify a

statistically significant difference and advocated the use of LCCRT for distal or bulky tumours.

SCRT does have the advantage of surgery occurring within ten days of completion of neo-adjuvant therapy in addition to reduced rates of acute toxicity, particularly with the absence of simultaneous chemotherapy, which was shown by both of the Polish and Australasian trials.(125,126) Compliance has also shown to be greater with SCRT due to the shorter length of therapy and therefore more convenient for patients, along with a decreased overall cost.(127) Although both the Polish and Australasian studies were unable to identify a greater rate of sphincter preserving procedures with the use of LCCRT, the German CAO/ARO/AIO larger study discussed in section 1.8.1, demonstrated both tumour downstaging (p <0.001), as well as higher rates of sphincter-sparing surgery (p = 0.004) when compared with post-operative chemoradiotherapy.(112)

The Stockholm III trial estimated to complete in January 2018, randomised patients between 1998 and 2010 to either SCRT followed by immediate surgery (within seven days), SCRT with delayed surgery (at four to eight weeks) or LCCRT with delayed surgery.(128) The study was powered to have a sample size of 840 patients with time to local recurrence as their primary endpoint. In their first interim analysis of 303 patients, significant differences were not seen between post-operative complications and reoperations between the three groups. Their most recent interim analysis compared the role of SCRT with immediate surgery vs. SCRT with delayed surgery.(129) Of the 462 patients analysed, complete pathological response was found to be significantly higher amongst patients undergoing SCRT with delayed surgery; 11.8% vs. 1.7% (p = 0.001).

The Berlin Rectal Cancer Trial began in 2004 with the aim of comparing SCRT with LCCRT within the context of a large multi-centre trial, with a target of 760 participants. (130) The trial is currently on-going and yet to publish their results. Based on the results currently available, it appears that LCCRT has an advantage in preventing local recurrence in locally advanced rectal cancer, but the results of both these upcoming trials should hopefully

provide confirmation as to the optimal pre-operative modality. Given the on-going debate over the more beneficial modality, current NICE guidance suggests the use of SCRT in patients with immediately operable tumours with a moderate risk of local recurrence where surgical margins are not threatened.(116) This ensures that surgical resection can yield clear margins and also reduce the potential spread of disease. In patients with tumours that are borderline between moderate to high risk, where resection margins are threatened, LCCRT is recommended with an interval prior to surgery, to ensure tumour response and shrinkage.

1.9 Adverse effects of radiotherapy

Although the aim of radiotherapy is to destroy cancer cells using ionising radiation, surrounding healthy cells are also damaged in the process leading to multiple adverse effects. The use of pelvic radiotherapy can therefore lead to a variety of regional and systemic side effects.

Peeters *et al* followed up 597 patients with a median follow-up of 5.1 years to assess the long-term side effects associated with neo-adjuvant short course radiotherapy, prior to total mesorectal excision.(131) Their study found a significantly increased bowel frequency in patients undergoing preoperative radiotherapy compared with those undergoing surgery alone (3.69 vs. 3.02 times/day; p = 0.011), as well as an increased incidence in the irradiated group of faecal incontinence both in the day (p < 0.001) and at night (p = 0.001). Anal mucus and blood loss was again found to be significantly higher in the group receiving pre-operative radiotherapy (p = 0.004 and p = 0.005 respectively).

Although bladder dysfunction is a commonly reported adverse effect of radiotherapy, this study did not identify any significant difference in urinary function between the two groups. Other significant long-term side effects that have been noted as a result of pelvic radiotherapy include lethargy, osteoporosis, lymphoedema, sexual dysfunction and infertility, as well as many others.(132,133) There is also variability in the severity and frequency reported of these adverse effects amongst patients. Bruheim *et al* investigated the long-term toxicity after receiving radiotherapy and undergoing TME and compared their outcomes with patients undergoing surgery alone.(134) The study assessed 535 patients between 1993 and 2003. Effects were evaluated at a median time of 4.8 years after surgery. It was shown that patients undergoing radiotherapy had significantly increased bowel frequency and faecal incontinence, with an overall negative impact on quality of life. There are several criticisms of this study including the observational nature of the study, the variable use of pre-operative and post-operative radiotherapy, and the time period over which it was conducted, during which the role of pre-operative radiotherapy was identified to be preferential. However despite these flaws, it demonstrates the significance of these adverse effects on patient lifestyle. Therefore, if radiotherapy could be used in a more selective manner, so that if a patient was predicted to have a clinical response, they would not be exposed to these potential toxic effects. Conversely, where patients are predicted to not have a clinical response to radiotherapy, they may be able to progress directly to surgery if their tumour was deemed to be resectable. This would prevent the potential delay in receiving a definitive treatment and prevent unnecessary potential adverse effects.

1.10 Assessment of response to neo-adjuvant therapy

After surgical resection, histopathological examination provides definitive staging of the rectal cancer and a regression grade is also assigned. Regression grade has been shown to be significantly associated with prognosis in multiple studies.(135–138)

1.10.1 Tumour regression grading

The two main systems used are Mandard(135) and Dworak(136). Mandard's system was initially shown to be prognostic in patients with oesophageal cancer treated with pre-operative chemoradiotherapy and has been subsequently adapted for use in the classification of rectal cancer.

Dhadda *et al* assessed whether the use of the Mandard scoring system was prognostic in assessing pathological tumour response following neoadjuvant chemoradiotherapy.(138) The adapted Mandard grading system used in this study is a paradoxical score to that used in Dworak's grading system (Appendix 1). The lowest Mandard and the highest Dworak grades denote absence of response to neo-adjuvant therapy and conversely the highest Mandard and lowest Dworak grades denote complete response with fibrosis. A cohort of 175 patients with locally advanced rectal cancer, all deemed to be inoperable or of borderline resectability due to a possible positive CRM, were either subjected to neo-adjuvant radiotherapy or chemoradiotherapy. Of the 158 patients that underwent resection, 22 (14%) were TRG 1, 65 (41%) were TRG 2, 49 (31%) were TRG 3, 20 (13%) were TRG 4, and 2 (1%) TRG 5. Use of Kaplan-Meier survival curves demonstrated that TRG was related to disease free survival (p < 0.0001) and overall survival (p = 0.012).

1.10.2 Modified tumour regression grade

When nodal status and Mandard score were analysed together in Dhadda's study, they were found to be strongly prognostic.(138) Therefore, a modified tumour regression grade including nodal status was proposed and was shown to be predictive of disease free survival (p < 0.0001) and overall survival (p < 0.0001) (Table 6). This modified system was used to grade clinical response in our study. Abdul-Jalil *et al*'s study of 153 patients was unable to find a significant correlation of disease-free survival with tumour regression grade.(139) However, they noted significance between complete pathological response and nodal status with prediction of long-term survival with 23.5% of patients demonstrating complete pathological response shown to have a five-year disease-free survival of 100% (p = 0.003). The authors acknowledged that this lack of statistical significance with tumour regression grading may be simply be due to their relatively small number of patients.

Modified tumour	Histopathology		
regression grade			
TRG 1	Complete response with absence of residual cancer and		
	fibrosis extending through the wall		
TRG 2	Presence of residual tumour cells scattered through the		
	fibrosis		
TRG 3	Increase in the number of residual cancer cells with		
	fibrosis prominent		
TRG 4	Residual cancer outgrowing fibrosis		
TRG 5	Macroscopic tumour; absence of regressive changes; any		
	node positive within irradiated volume		

Table 6) The modified tumour regression grade used by Dhadda et al(138)

1.11 Role of prediction of response to adjuvant therapy

Neo-adjuvant LCCRT has been shown to result in both complete clinical response, where the residual primary tumour cannot be detected clinically or complete pathological response, where no viable tumour cells are evident on histopathological examination after resection. The rate of response has been reported to range from 8%(140) to 30.5%(141).

Habr-Gama et al followed up patients eight weeks after completion of neoadjuvant LCCRT and if they were deemed to have a complete clinical response, further follow-up was arranged on a monthly basis rather than proceeding to an immediate operation.(142) Response was assessed clinically, radiologically and endoscopically, with biopsies being performed during proctoscopy. Detection of a significant residual ulcer or biopsies positive for cancer were termed as having an 'incomplete clinical response'. In their study of 265 patients, 71 (26.8%) patients were found to be complete clinical responders (no cancer identified clinically or radiologically). These patients were followed up for a mean time of 57.3 months (range 12-156 months). Two of these patients developed endoluminal recurrence and three developed systemic metastases. None of the patients developed pelvic recurrence. Of the 194 patients deemed to have an incomplete clinical response after radical surgery, 22 (8.3%) patients were actually shown to have undergone complete pathological response. Mean follow-up in this group was 48 months (range 12-83). Three (13.6%) patients in the resection group with complete pathological response developed systemic metastases and two (9.1%) patients within this group died of the disease.

Due to none of the patients in the observation group dying of cancer-related causes, five-year overall survival was shown to be higher in this group vs. the resection group; 100% vs. 88% respectively (p = 0.01). Disease free survival was not found to be significantly different between the two groups; observation group, 92% vs. resection group, 83% (p = 0.09). Again no statistical difference was seen in recurrence and mortality rates between the two groups in the management of complete responders (p = 0.2). The authors thus advocate following a strict algorithm where a course of non-operative management can be taken to limit radical surgery. During these monthly follow-up appointments, physical and digital rectal examinations were performed along with proctoscopy, with biopsies when possible. Additionally serum carcinoembryonic antigen (CEA) levels, a tumour marker associated with recurrence of colorectal cancer, were also taken.(143,144) During their first year of follow-up, patients underwent sixmonthly CT scans of the abdomen and pelvis with chest radiographs. Follow-up appointments during the second and third year were two and six monthly respectively. In the event of distal metastases without local recurrence, patients were treated only for metastatic disease. Patients with incomplete or no response underwent immediate surgery. Even in the event of local recurrence, Habr-Gama *et al* demonstrated that salvage surgery was possible with little evidence to suggest that this delay in surgery led to worse outcomes.(145–147)

Unfortunately since Habr-Gama's pioneering work, only a few other authors have adopted a watch and wait algorithm with similarly successful outcomes.(148,149) Most recently, the Danish Colorectal Cancer Group study, NCT00952926, carried out a prospective study within a single centre between 2009 and 2013 to evaluate the role of watchful waiting.(150) The patients were given a brachytherapy tumour boost in addition to LCCRT (combined prescribed radiation dose of 66Gy); a regimen not used by other

studies as standard. Of their 51 eligible patients, 40 patients (78%) had a complete clinical response and underwent a median observation follow-up of 23.9 months.(150) Cumulative local recurrence at one year was 15.5% (95% CI 3.3-26.3) and 25.9% (95% CI 9.3-42.8%) at two years. With the heterogeneity in treatment regimens, it is difficult to compare studies directly, but it appears that there is growing evidence of success using this treatment strategy. Further prospective multicentre studies are required to confirm the reproducibility of these results in addition to long-term outcomes. There are several prospective trials currently taking place including the NCT01047969 Royal Marsden study due to complete in June 2019.(151) Along with this, the International Watch & Wait database has been set up by the European Registration of Cancer Care, and the Champalimaud foundation to assess the long-term outcomes of patients undergoing watchful wait strategies for rectal cancer.(152) Although TME has been shown to highly effective in preventing local recurrence, it is also associated with significant morbidity and mortality, with a 30-day operative mortality 5.0% (range 3.7-6.8) for anterior resections and 6.8% (range 4.1-10.6) for APER's.(153) Currently the administration of neo-adjuvant therapy is guided by radiological and histological determination of stage and grade, but these attempts to predict tumour behaviour are less than accurate.(154,155) With complete response rates of greater than 30%, it is likely that a substantial proportion of patients may unnecessarily receive radiotherapy with minimal benefit. If clinicians had the ability to predict tumour response to neo-adjuvant therapy, it could potentially be administered in a more selective and effective manner. This would avoid the potential for chemoradiotherapy related side effects and toxicity in patients who would not clinically benefit and surgery could be performed without delay. In patients where complete response to neo-adjuvant therapy is predicted, the nature of the surgical procedure offered could also be altered, to either perform a local or less radical resection of the rectal cancer or to even avoid surgery and subject patients to regular follow-up.

The heterogeneity in tumour response is likely to be due to a combination of factors including tumour size, location, differentiation, as well as biological traits linked to the individual.

1.12 Radiation induced cell death

The purpose of radiotherapy is to deliver high-energy radiation to ionise tissue, which leads to DNA damage and thus destroy cancer cells.(156–158) In the neo-adjuvant treatment of rectal cancer, this is delivered as external beam radiotherapy, whereby focused photon beams, generated by a linear accelerator, are targeted at the area of concern.(118) Radiation can lead to DNA damage either by producing secondary charged particles or indirectly by inducing the formation of free radicals within the nucleus, leading to damage to adjacent cells.(156,159) These free radicals can be formed by oxidation of water or from the formation of secondary partially reactive oxygen species. Although the aim of radiotherapy is to target cancerous tissue, adjacent healthy tissue is also affected and this can potentially lead to adverse effects as discussed in section 1.9.

Although single and double stranded DNA breaks are induced by irradiation, double stranded breaks are considered to lead to cell death in conjunction with damage of cell membranes.(156,160,161) In the event of irreparable damage to all tumour cells, sterilisation occurs, where further cell proliferation cannot occur, which is necessary for complete pathological response. However, in partial sterilisation, stasis of the tumour or regression may occur, which although may lead to clinical response, may result in regrowth from cells still able to proliferate. The aim of radical radiotherapy is to induce complete sterilisation of all tumour cells with minimal damage to adjacent healthy cells. Tumour response to fractionated radiotherapy is thought to be controlled by five factors: radiosensitivity, repair, repopulation, re-oxygenation and redistribution; of which radiosensitivity is thought to be most important (Table 7).(161–163)

Table 7) Table to demonstrate the role of radiobiological factors in the response of malignant tumours in response to fractionated radiotherapy. Adapted from Practical Radiotherapy Planning(161) and Text book of radiotherapy(163).

Factor	Mechanism	Clinical significance
Radiosensitivity	Variation in sensitivity of	Variable pathological
	malignant and normal cells in	response
	response to radiotherapy.	
Repair	Variation in ability of cells to	Fractionated therapy requires
	repair DNA damage with	a greater total dose to ensure
	repair being more effective in	complete cell death. During
	non-proliferating cells.	these intervals, injured
		normal tissue can also be
		repaired.
Repopulation	Surviving malignant cells	Accelerated therapy, where
	proliferate more rapidly after	treatment times are
	initiation of treatment.	shortened may be
		advantageous.
Re-oxygenation	Hypoxia, due to temporary	The surviving hypoxic cells
	vessel constriction and	re-oxygenate to become
	outgrowth of vessels, which	radiosensitive. This reinforces
	tend to occur in tumours, to	the role of fractionation of
	make them less responsive to	radiotherapy.
	radiotherapy.	
Redistribution	Cells in certain phases of the	Fractionated treatment allows
	proliferative cycle (e.g. late S	cells to be targeted at various
	phase) are radioresistant,	points during the cell cycle.
	whereas those undergoing	
	mitosis are relatively	
	radiosensitive.	

1.12.1 Mechanisms of radiation induced cell death

There have previously thought to be two processes that contribute to cell death due to radiation: apoptosis and necrosis.(163) Apoptosis is the active process of programmed cell death, whereas necrosis is a passive process resulting from the attempted mitosis of cells containing unrepaired DNA breaks and lethal chromosomal abnormalities. Apoptosis has been shown to be the main mechanism of cell death in irradiated haematopoietic cell lines, occurring immediately after cell division and in addition, during interphase, occurring within hours of irradiation.(160,164) However, despite this, due to

the loss of pro-apoptotic mechanisms in the majority of solid tumours, apoptosis does not play as great a role and therefore other modalities such as mitotic catastrophe, senescence and autophagy are thought to be responsible.(158,165)

1.12.1.1 Apoptosis in response to ionising radiation

Apoptosis is characterised by pykinosis, cell condensation/shrinkage and internucleosomal breakage of chromatin.(166,167) As described in section 1.2.3.3, mutation of the tumour suppressor p53 has been linked to radioresistance, with p53 shown to be responsible for rapid interphase apoptosis after irradiation.(165) This radiation induced p53 dependent apoptosis has been shown to occur within a few hours as a pre-mitotic event.

Cleavage of caspase substrates has also shown to be essential in the process of apoptosis in response to either external or internal stimuli.(168) The caspase cascade is activated by radiation-induced apoptosis via two main pathways, an intrinsic (mitochondrial) or an extrinsic (death receptor) pathway, both leading to the activation of effector caspases (caspase-3, caspase-6 and caspase-7), which are responsible for apoptotic execution.(169,170) Although initially thought to be mutually exclusive, these two pathways have been shown to converge.(171,172) The intrinsic pathway acts through caspase-9 and the extrinsic pathway acts through caspase-8, with both pathways activating the effector caspases (Figure 9a).(173) An additional pathway involves T-cell mediated mediated cytotoxicity and perforin-granzyme-dependent killing of the cell, with induction of this pathway via either granzyme A or B (Figure 9b).(174)



Figure 9a) Simplified diagram of caspase extrinsic and intrinsic pathways adapted from Hipfner *et al.*(175) APAF 1 – apoptosis activating factor 1. IAP – inhibitor of apoptosis.



Figure 9b) Simplified diagram of the perforin-granzyme pathway as an alternate method of apoptosis adapted from Lieberman 2003 and Pinkoski and Green.(176,177) GAAD – Granzyme A activated DNase

Intrinsic pathway

Mitochondrial outer membrane permeabilisation (MOMP) occurs via the intrinsic pathway and leads to disruption of mitochondrial function.(178) Bcl-2 and inhibitor of apoptosis (IAP) protein families are also involved in the cascade and these proteins are overexpressed in human cancers, leading to a failure in apoptosis. Bcl-2 proteins are believed to both inhibit and upregulate cytochrome c release to regulate the permeability of mitochondria. Inhibition occurs via proteins such as Bcl-2, Bcl-X and Mcl-1, whereas upregulation occurs via BAX and BID. Whether a cell undergoes apoptosis or survives is dependent on the overall balance of these pro- or anti-apoptotic proteins. A supramolecular caspase-activating complex is formed from cytochrome c and apoptosis activating factor 1 (APAF 1) which induces caspase 9. Capase 9 is cleaved to then activate the effector caspases. IAP proteins prevent capase 9 and other caspases from acting further downstream to prevent cell death.

Extrinsic pathway

The extrinsic pathway is also active in radiation-induced apoptosis and requires pro-apoptotic ligands to activate the transmembrane death receptors, including Fas, DR3, DR4 and DR5, which are members of the tumour necrosis factor (TNF) receptor gene superfamily.(179,180,174) The resulting ligand/receptor complexes allows further binding of cytoplasmic adapter proteins via their death domains to form a death-inducing signalling complex, which subsequently results in the activation of procaspase-8. Upon activation of caspase-8, effector caspases are triggered transducing amplified signals intracellularly that thus lead to cell destruction.

Perforin-granzyme pathway

Although the intrinsic, extrinsic and granzyme B pathways utilise the same execution pathway via caspase-3, granzyme A acts via a separate, caspaseindependent pathway.(181) In addition to acting via the Fas receptors in extrinsic pathway, cytotoxic T-lymphocytes also secrete granules containing perforin, a pore-forming protein that is able to create pores across plasma membranes.(182,183) Granzymes A and B, pro-apoptotic proteases, also secreted within these granules, are then able to pass into these target cells.(184)

Granzyme B has been demonstrated to act by cleaving proteins, including Bid, to lead to the activation of caspase 10 or acting directly on caspase-3 to initiate the execution pathway.(185) Granzyme A however leads to the production of reactive oxygen species, which cleave the SET complex, an endoplasmic reticulum-associated complex.(181,184,186) This in turn releases the inhibition of NM23-H1, a granzyme A activated DNase, leading to single stranded DNA damage and preventing further maintenance.

Use of fractionated radiotherapy has been shown by several authors to promote overall greater levels of apoptosis than a large single dose.(187– 189) These studies suggested that apoptosis is cumulative in relation to the number of fractions delivered.

1.12.1.2 *Mitotic catastrophe in response to ionising radiation*

Mitotic catastrophe occurs due to aberrant segregation of chromosomes leading to the formation of giant cells and is now considered to be the foremost mechanism by which solid malignant tumours response to radiotherapy occurs.(165,190,191) In response to DNA damage, cell cycle arrest tends to occur in G2 phase to allow apoptosis or senescence to follow, however when this is not possible, mitosis occurs prior to completion of DNA repair.(192) These cells have been noted to have aberrant nuclear morphology, multiple nuclei and several micronuclei. This mechanism of cell death has been noted to occur several days after the induction of radiotherapy.

Although the mechanisms by which mitotic catastrophe are not fully understood, several suggestions have been put forward. Firstly faulty checkpoints in the cell cycle have been identified, permitting faulty progression into mitosis before DNA damage has been repaired (Figure 10).(190,193) This has been found to be associated with p53 mutation.



Synthesis phase

Figure 10) Diagrammatic representation of the cell cycle disruption in mitotic catastrophe with inactivation of the G2/M checkpoint, permitting early entry into mitosis of cells with damaged DNA that has not yet been repaired. Adapted from Molecular Biology of the Cell.(194)

Secondly amplification of centrosomes has been identified as a mechanism by which mitotic catastrophe can occur.(195–197) Normally a centrosome acts as the major microtubule organising centre of the cell and is duplicated during the cell cycle. During mitosis, the two centrosomes form the poles of the mitotic spindle, dividing the chromosomes into daughter cells. This error leads to mitotic spindles with multiple poles to form cells with abnormal chromosome separation and multiple nuclei. There have been suggestions that mitotic catastrophe is not a sole mechanism of cell death, and actually works in conjunction with and acts as a precursor to other mechanisms such as apoptosis, necrosis and senescence.(190,191,193) Cell death via necrosis or apoptosis may not occur immediately due to cells continuing through the cell cycle repeatedly leading to aneuploidy or polyploidy.

1.12.1.3 Necrosis in response to ionising radiation

Necrosis unlike apoptosis described in section 1.12.1.1 has been defined morphologically by swelling of cellular organelles and loss of plasma integrity.(167,198) This leads to the influx of extracellular ions and osmotic shifts of fluid. It has been previously referred to as being uncontrolled and pathological, however there is growing evidence to suggest that it is actually a controlled event as discussed by several authors.(199–201) Although necrosis is not believed to be a common mechanism of cell death in this setting, studies have shown that it is the predominant mechanism in response to high doses of radiation.(202–204) Therefore it is likely to only play a limited role in clinically applicable radiation schedules.

1.12.1.4 Senescence in response to ionising radiation

Senescence is the permanent loss of ability of cells to proliferate due to cell cycle arrest that occurs to remove irreparable DNA damage.(205,206) Despite this, these cells are still viable and continue to be metabolically active. Over time, normal healthy cells eventually stop replicating, due to progressive telomere shortening with each division reaching a critical point, as first described by Hayflick and Moorhead.(207) It was hypothesised that the evolutionary role of senescence was to prevent cancer.(208) Stress-induced premature senescence (SIPS) can occur in response to irradiation, where senescence is activated prematurely secondary to DNA damage.(205) A DNA damage response is induced in response to low doses of radiation, which identifies the DNA damage to activate cell cycle arrest to permit repair. Phosphorylation of sensors and effectors of this DNA damage response including p53 leads to the upregulation of cyclin dependent kinase inhibitor p21, which subsequently inhibits CDK2 kinase activity, thus

ensuring cell cycle arrest in G1. If DNA damage is difficult to repair, cell death (apoptosis or mitotic catastrophe) may be induced. However, a persistent DNA damage response can occur to induce to senescence. It is unclear however what guides a cell to undergo cell death or senescence.

1.12.1.5 Autophagy in response to ionising radiation

Autophagy is the catabolic process by which lysosomal degradation of cytoplasmic components and organelles occurs to generate energy and metabolites.(167,209) A phagophore is derived from the endoplasmic reticulum, Golgi apparatus and mitochondria and encloses cytoplasmic components and organelles to form an autophagosome, a double-membrane vesicle. This then fuses with a lysosome to create an autophagolysosome, within which acid hydrolases break down the contents. Autophagy has been identified by several studies as an alternative modality of cell death following radiotherapy, however, its exact role has not been yet fully identified.(209–212)

1.13 Biomarkers predicting response to neo-adjuvant chemoradiotherapy

Despite the abundance of literature examining potential biomarkers to predict response to neo-adjuvant therapy, to this date, none have been validated. Kuremsky *et al*'s literature search was followed, as this was the largest review article to date in which 1,204 articles were retrieved via using PubMed[™].(213) They evaluated molecular biomarkers for response to neo-adjuvant chemoradiotherapy in rectal cancer and initially identified 36 'putative' biomarkers. However, only markers with more than five studies in the literature were chosen to focus upon: these were p53, EGFR, thymidylate synthase (TS), Ki-67, p21 and bax/bcl-2. More recent studies performed subsequent to this paper have also been included.

1.13.1 Analysis of p53

Mutated p53 in malignant cells has been previously associated with resistance to chemoradiotherapy and conversely, the presence of wild-type p53 in malignant cells has been shown by several authors to be associated with response to chemoradiotherapy.(214,215) The role of mutant p53 is discussed in section 1.2.3.3. Immunohistochemical analysis indicates the presence of mutated p53 expression, whereas tissue with wild-type (normal) p53 is not stained. A summary of the studies evaluated demonstrating a significant correlation between p53 expression and response to LCCRT is demonstrated in table 8.

In Kuremsky's review, twenty-one studies were identified that evaluated p53 as a biomarker for rectal cancer response to neo-adjuvant chemoradiotherapy.(213) However, only four of these studies found any significant correlation between p53 expression and any survival or outcome measure.(216–219)

Subsequent to Kuremsky's qualitative review in 2008, Chen *et al* performed a meta-analysis to evaluate p53 status as a predictive biomarker in response to neo-adjuvant therapy, including 30 studies (up to 8 May 2012) containing 1830 patients.(220) Only 25 of these studies used protein detection using immunohistochemistry, with others using gene detection. Wild-type p53 status was significantly associated with a good response, defined as residual tumour rate of <50% (RR = 1.30; 95% CI = 1.14-1.49; p < 0.001), as well as complete pathological response (RR=1.65; 95% CI=1.19–2.30; p=0.003). Although there was heterogeneity between the studies included, this study included a large number of cases with no evidence of publication bias detected. However the role of immunohistochemical detection of p53 as a potential biomarker remains yet to be proven.

Since Chen's review up to 1 November 2015, using PubMed[™] to search for studies involving the terms rectal cancer and p53, 59 studies were identified. Of these studies, only eight evaluated p53 status in relation to neo-adjuvant therapy, with only one of these studies demonstrating any

significant correlation (i.e. a *p* value < 0.05) between p53 expression and any survival or outcome measure.

Hur *et al* performed a retrospective analysis of 81 patients with locally advanced rectal cancer, evaluating complete pathological response after neo-adjuvant chemoradiotherapy in relation to p53 status.(221) Immunohistochemical analysis of biopsies taken prior to neo-adjuvant therapy was used to assess p53 status. Of the 32 patients with a low expression of p53, 15 (46.9%) were found to have a complete pathological response to chemoradiotherapy, whereas only 12 of the 49 (24.5%) with high expression of p53 were found to have a complete pathological response (p = 0.03). Multivariate analysis also demonstrated p53 to be an independent predictor of complete pathological response (p = 0.04). Although this study did note a significant correlation between low p53 expression and complete pathological response, there are several limitations including the retrospective nature of the study, relative small numbers and a potential selection bias of only including patients in whom sufficient tissue was available for analysis, thus leading to an overall complete pathological response rate of 33.3%. The authors acknowledge that use of a scoring system involving assessment of multiple biomarkers may be preferential as a predictive tool.

Immunohistochemical p53 positive staining has been found to be discordant with mutation when compared to genetic analysis and therefore its use as a biomarker may be limited.(222) Given the relatively few studies, in addition to the relatively small numbers interrogated that report an association and also studies showing a contrary relationship, it is unlikely that immunohistochemical detection of p53 could serve as a realistic predictor of response to neo-adjuvant therapy.

Author(s)	Year of	No of	LCCRT	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	regimen		
Spitz et	1997	42	45Gy + 5-	Pathological response	10/19 patients with p53 negative tumours
al(216)		patients	FU		achieved pCR vs. 3/23 patients with mutated
					p53 (<i>p</i> = 0.02)
Luna-Perez et	1998	26	45Gy + 5-	Residual tumour rate	2/12 patients with p53 negative tumours
al(217)		patients	FU		identified to have >50% residual tumour vs.
					10/14 with mutated p53 (<i>p</i> = 0.018)
				Sphincter preservation rates	8/12 patients with p53 negative tumours
					underwent sphincter preserving procedures vs.
					2/14 with mutated p53 (<i>p</i> = 0.01)
Esposito et	2001	38	45-50.4Gy	Pathological response	Patients with mutated p53 tumours were found
al(219)		patients	+5-FU		to be more predictive of response on
					multivariate analysis ($p = 0.03$)
Lin <i>et al</i> (218)	2006	70	45Gy + 5-	Fair response defined as complete	Patients with p53 negative tumours were more
		patients	FU	regression or tumour <i>in situ</i> in	likely to have a fair response ($p = 0.006$)
				histopathology	

 Table 8)
 Table summarising the studies analysed that demonstrated a correlation between p53 analysis and response to LCCRT

Author(s)	Year of	No of patients	LCCRT regimen	Significant study	Summary of significant outcome(s)
	publication			endpoint	
Chen et	2012	Meta-analysis	Heterogeneity	Good response defined	Patients with p53 negative tumours were
al(220)		(30 studies	between studies	as residual tumour rate	more likely to have a good response (<i>p</i> <
		containing 1830		of <50%	0.001)
		patients		Pathological response	Patients with p53 negative tumours were
					also more likely to achieve pCR
Hur et	2014	81 patients	45Gy +5.4Gy boost	Mandard regression	15/32 patients with tumours with low p53
al(221)			+5-FU/leucoverin	grade	expression were found to have pCR vs. 12/49
					with high p53 expression ($p = 0.03$)

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; pCR – complete pathological response)

1.13.2 Analysis of Epidermal growth factor receptor

EGF and related ligands stimulate the receptor (EGFR) to initiate multiple activities, including mitogenesis, apoptosis and differentiation. EGFR has been identified as a proto-oncogene and is associated with the development of a variety of cancers.(223) An inverse correlation has been charted between the magnitude of EGFR expression and radio-curability using murine models.(224,225) A summary of the studies evaluated demonstrating a significant correlation between EGFR expression and response to LCCRT is demonstrated in table 9. Kuremsky's review article identified five papers where the role of EGFR expression was correlated with neo-adjuvant chemoradiotherapy in locally advanced rectal cancer.(213,226–230)

Subsequent to Kuremsky's review, Spolverato *et al* performed their systematic review of potential predictive factors of response to neoadjuvant chemoradiotherapy.(231) In addition to the studies identified by Kuremsky, four further studies were identified, only two of which identified a correlation.(232,233)

PubMed^m was used to search for studies up to 1 November 2015 performed subsequent to Spolverato's review involving the terms rectal cancer and EGFR. This search identified 81 studies. Of these studies, only nine evaluated EGFR status in relation to neo-adjuvant therapy, with only one of these studies demonstrating any significant correlation (i.e. a *p* value < 0.05) between EGFR expression and any survival or outcome measure.

Several studies have reviewed changes in EGFR expression between pretreatment biopsies and resection specimens after undergoing neo-adjuvant chemoradiotherapy.(234–236) Although overall survival and disease-free survival have been significantly associated with increased expression of EGFR between biopsies taken prior to and post neo-adjuvant therapy, its value as a potential predictive biomarker was not assessed. Zhao *et al* performed a systematic review and meta-analysis up to 30 March 2015 of EGFR polymorphisms with TRG in response to pre-operative chemoradiotherapy.(237) Eleven articles were deemed eligible for their study, eight of which were carried out in rectal cancer studies, the remainder assessing oesophageal cancer. Three of the studies assessed the polymorphism EGFR G497A amongst (n = 634) and four studies assessed EGFR CA repeat polymorphism (n = 396). However, meta-analysis of these studies did not identify a correlation with tumour response, even on subgroup analysis of rectal cancers.

Of these studies, only Kim's(227), Giralt's(226) and Pei's(238) groups demonstrated an association between immunohistochemical EGFR status and treatment outcome, which suggests that measuring EGFR status quantitatively, rather than qualitatively as positive or negative, may be of greater benefit. Single nucleotide polymorphisms of the regulatory EGFR promoter region identified using PCR was shown by several studies to act as a potential biomarker, but will require further evaluation.

Author(s)	Year of	No of	LCCRT regimen	Significant study	Summary of significant outcome(s)
	publication	patients	(unless otherwise	endpoint	
			stated)		
Giralt et	2005	87	45Gy (+5.4Gy boost in 8	Pathological response	2/12 patients with tumours with positive EGFR
al(226)		patients	patients) +		expression (≥5%) achieving pCR vs. 8/35 patients
			chemotherapy (n = 50)		with negative expression ($p = 0.006$).
			5-FU + leucoverin (n =	Disease-free survival	Disease-free survival was noted to be significantly
			33) or		shorter in patients with tumours with negative EGFR
			Tegafur-uracil +		expression ($p = 0.03$)
			leucoverin (n = 17)		
Kim et	2006	183	50.4Gy + 5-FU +	Tumour downstaging	Using a multiple regression model, a low level of EGFR
al(227)		patients	leucoverin (n = 94) or	defined as reduction	expression (staining and extension) was a significant
			capecitabine +	of at least one T-stage	predictor for increased tumour downstaging (p =
			leucoverin (n = 89)		0.012)
Spindler et	2006	63	60Gy + 5Gy boost	Mandard TRG	Using PCR to assess the Sp1 binding site of the
al(228)		patients	+tegafur-uracil +		regulatory EGFR promoter region, 10/29 (34%) GG
			leucovorin		homozygote patients were found to have a major
					response defined as TRG 1 or 2 vs. 22/34 (65%) with
					GT heterozygosity or TT homozygosity

 Table 9)
 Table summarising the studies analysed that demonstrated a correlation between EGFR analysis and response to LCCRT

Author(s)	Year of	No of	LCCRT regimen	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	(unless otherwise		
			stated)		
Spindler et	2007	60	60Gy + 5Gy boost	Mandard TRG	Using PCR to assess a combination of
al(229)		patients	+tegafur-uracil +		polymorphisms, TS 2/2 and EGFR Sp1-216 or
			leucovorin		EGF A61G heterozygosity was found to be
					predictive of pCR ($p = 0.01$)
Toiyama et	2010	40	SCRT 20Gy + 5-FU	Tumour regression grading	Using PCR, low levels of EGFR expression were
al(232)		patients	+tegafur-uracil	based on Japanese Research	associated with a high rate of tumour
				Society for Cancer of the	regression ($p = 0.013$)
				Colon and Rectum	
				3-year disease free-survival	3-year disease-free survival found to be
					significantly higher in in patients with high
					levels of expression (90% vs. 70%; <i>p</i> = 0.003)
Bengala <i>et</i>	2009	40	Cetuximab (planned	Dworak TRG	High EGFR gene copy number associated with
al(233)		patients	doses completed in n =		high TRG ($p = 0.0016$)
			28)+ 50Gy (n =		Note findings not replicated in authors follow
			33)/50.4Gy (n = 7)		up study using chemotherapy regimen without
					cetuximab(239)
Pei et	2014	44	Unknown	TNM downstaging and TRG	Significantly higher percentage of downstaging
al(238)		patients			and TRG 3 and 4 in patients with low
					immunohistochemical EGFR expression (p <
					0.01)

(5-FU – 5-Fluorouracil; EGFR – epidermal growth factor receptor; G – guanine; LCCRT – Long course chemoradiotherapy; pCR –

complete pathological response; PCR – polymerase chain reaction; T – thymine; TRG – tumour regression grade)

1.13.3 Analysis of Thymidylate synthase (TS)

TS acts as a catalyst in a fundamental stage of DNA biosynthesis and has been shown to be a target for fluoropyrimidines, including 5-FU (Figure 11).(240–242) Multiple authors have demonstrated a poorer response of tumours with overexpression of TS in relation to 5-FU based chemotherapy regimens and also poor prognosis.(243–245) A summary of the studies evaluated demonstrating a significant correlation between TS expression and response to LCCRT is demonstrated in table 10. Kuremsky's review identified 6 papers that identified a significant relationship between TS and treatment outcome.(213,229,246–250)



Figure 11) Simplified diagram demonstrating the effect of 5-FU, which is converted intra-cellularly to cytotoxic metabolites. These metabolites can either inhibit TS directly or become incorporated into DNA and RNA molecules. TS acts as a catalyst in the conversion of dUMP to dTMP, but metabolites of 5-FU bind to the nucleotide binding site of TS, leading to an imbalance of deoxynucleotides and increased levels of dUTP (not shown), which in turn lead to both RNA and DNA damage. Adapted from Longley *et al* and Weiss *et al.*(242,251) (5-FU – 5-flurouracil; dUMP – deoxyuridine monophosphate; dTMP – deoxythymidine monophosphate; dUTP – deoxyuridine triphosphate; TS – thymidylate synthase)

In addition to these studies, Spolverato *et al*'s systematic review also identified a further four studies demonstrating a significant correlation between TS expression and response to neo-adjuvant chemoradiotherapy.(231,252–255)

Of the studies that found a significant relationship between TS expression using either immunohistochemistry or PCR with clinical outcome, five demonstrated improved outcomes with low or absent TS expression, whereas three studies produced contradictory results. It was noted by Spolverato *et al* that oxiliplatin downregulates TS expression, which may account for two of these contradictory results.(256,257) Three studies evaluated TS gene promoter polymorphisms, however, these studies also produced variable results with two of the studies demonstrating homozygosity of triple tandemly repeated sequences to be associated with lower tumour response. Given these variable results, it is unlikely that TS is likely to prove to be beneficial as a predictive biomarker.

Author(s)	Year of	No of	LCCRT regimen (unless	Significant study	Summary of significant outcome(s)
	publication	patients	otherwise stated)	endpoint	
Okonwko et	2001	25	45Gy + 5-FU	pCR	Immunohistochemical TS overexpression was
al(246)		patients			associated with poor response in 7/13
					unresponsive tumours vs. 1/12 responsive
					tumours. Note statistical significance not
					demonstrated.
Villafranca	2001	65	45-54Gy+ 5-FU	Tumour	Patients found to be homozygous for triple
et al(247)		patients	+carboplatin/oxiliplatin or 45-	downstaging	tandemly repeated sequences of the TS
			54Gy + tegafur-uracil +		polymorphism were demonstrated to have a lower
			leucovorin		probability of downstaging than those with double
					tandemly repeated sequences or heterozygotes (<i>p</i> =
					0.036)
Saw et	2003	60	45Gy/50.4Gy +/- 5-FU +	Tumour	Tumours not staining for TS found to be predictive
al(248)		patients	leucoverin	downstaging	of downstaging with chemoradiotherapy 7/10 vs.
			(Radiotherapy only n = 25;	defined as a	TS positive staining $8/25$ ($p = 0.047$), however, no
			chemoradiotherapy n = 35)	decrease in T-	significance noted with tumours treated solely with
				stage	radiotherapy.

Table 10) Table summarising the studies analysed that demonstrated a correlation between TS analysis and response to LCCRT

Author(s)	Year of	No of	LCCRT regimen	Significant	Summary of significant outcome(s)
	publication	patients	(unless otherwise	study	
			stated)	endpoint	
Jakob et	2004	14	50.4Gy + 5-FU	Dworak TRG	Low TS gene expression using PCR found to be predictive of
al(258)		patients			regression (TRG 2-4) (<i>p</i> = 0.0179)
Jakob et	2005	25	50.4Gy + 5-FU	Dworak TRG	High TS expression using immunohistochemistry found to be
al(259)		patients			associated with lack of tumour response (TG 0-1) ($p = 0.04$)
Jakob et	2008	22	50.4Gy + 5-FU	Dworak TRG	Low TS gene expression using PCR found to be predictive of
al(249)		patients			regression (TRG 2-4) (<i>p</i> < 0.05)
Negri <i>et</i>	2008	57	40Gy (n = 38)/45Gy +	pCR	No correlation in patients treated solely with radiotherapy.
al(250)		patients	5-FU + oxiliplatin (n =		High TS immunostaining levels correlated with greater
			19)		response in patients treated with chemoradiotherapy (<i>p</i> =
					0.015)
Spindler et	2007	60	60Gy + 5Gy boost	Mandard	Using PCR to assess TS polymorphisms, patients who were
al(229)		patients	+tegafur-uracil +	TRG/pCR	homozygous for triple tandemly repeated sequences were
			leucovorin		less likely to have a pCR when compared to those with a
					double repeat or heterozygotes ($p = 0.048$)
Kikuchi et	2009	60	45Gy + S1 (tegafur-	Dworak TRG	High levels of TS correlated with responders (classed as TRG
al(252)		patients	uracil + gimeracil		3 and 4) in both univariate (p < 0.05) and multiple logistic
			+oteracil) +		regression ($p = 0.019$) analyses
			irinotecan		
Carlomagno	2010	43	45Gy + capecitabine +	pCR	High levels of TS correlated with pCR ($p = 0.002$)
et al(253)		patients	oxiliplatin		

Author(s)	Year of	No of	LCCRT regimen (unless	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	otherwise stated)		
Páez et	2010	51	45Gy + 5-FU/capecitabine	Pathological response (with	Triple tandemly repeat sequences of the
al(254)		patients	(variable regimens of	responders classed as pCR or	5'-UTR region were found to be
			chemotherapy used)	presence of residual microfoci of	associated with higher response rates (p
				carcinoma) and overall survival	= 0.013) and greater overall survival $(p =$
					0.037)
Hur et	2011	44	45Gy + 5-FU	Tumour downstaging defined as	Single nucleotide polymorphism of the TS
al(255)		patients		a decrease in T-stage	enhancer region (guanine to cytosine)
					exhibited a greater rate of tumour
					downstaging ($p = 0.001$)

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; pCR – complete pathological response; PCR – polymerase chain

reaction; TS – thymidylate synthase; TRG – tumour regression grade)

1.13.4 Analysis of Ki-67

The Ki-67 antigen is present in all active phases of the cell cycle, but is absent from resting cells (cell cycle phase G0 – see Figure 10) and therefore detection using monoclonal antibodies has been used as a marker of proliferation. The Ki-67 labelling index (the fraction of tumour cells positive for Ki-67) has been correlated with disease prognosis in prostate and breast carcinomas.(260) Kuremsky *et al* were only able to identify two studies demonstrating a significant association between Ki-67 as biomarker and clinical outcomes in rectal cancer.(213,249,261) A summary of the studies evaluated demonstrating a significant correlation between Ki-67 expression and response to LCCRT is demonstrated in table 11.

Spolverato *et al* were only able to identify an additional two studies to demonstrate a significant correlation.(231) Kikuchi *et al's* study also demonstrated that high immunohistochemical labelling indices of Ki-67 correlated with clinical response to neo-adjuvant therapy on both univariate (p < 0.05) and with the use of multiple logistic regression (p = 0.002) analyses.(252) Huerta *et al* subsequently used a tissue microarray construct and used a panel of immunohistochemical markers.(262) They identified Ki-67 labelling index to be significantly lower in good responders on univariate analysis (p < 0.001).

Given the current lack of evidence and conflicting associations in relatively small studies, the role of Ki-67 as a future biomarker seems doubtful.

Author(s)	Year of	No of	LCCRT regimen	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	(unless otherwise stated)		
Kim <i>et</i> <i>al</i> (261)	2001	23 patients	45-54Gy + 5-FU + leucovorin	Pathological response classed as complete (no residual tumour), partial (tumour volume diminished over 50% and/or downstaging) or no response	Ki-67 labelling index found to be significantly higher in patients that underwent partial or complete response vs. no response ($p = 0.029$)
Jakob <i>et</i> <i>al</i> (249)	2008	22 patients	50.4Gy + 5-FU	Dworak TRG	Responders (TRG 2-4) demonstrated a significantly lower Ki-67 expression than non-responders (<i>p</i> < 0.05)
Kikuchi et al(252)	2009	60 patients	45Gy + S1 (tegafur- uracil + gimeracil +oteracil) + irinotecan	Dworak TRG	High labelling indices of Ki-67 correlated with responders (classed as TRG 3 and 4) in both univariate ($p < 0.05$) and multiple logistic regression ($p = 0.002$) analyses
Huerta <i>et</i> al(262)	2010	38 patients	50.4Gy + capecitabine	Response (classified as good being >50% pathological response and poor as <50%)	Ki-67 labelling index significantly lower in good responders

Table 11) Table summarising the studies analysed that demonstrated a correlation between Ki-67 analysis and response to LCCRT

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; pCR – complete pathological response; TRG – tumour regression

grade)

1.13.5 Analysis of p21

Although p21 is a tumour suppressor, it can behave as an oncogene by suppressing apoptosis and promoting the assembly of type-D cyclins, which within the cell cycle are initiated during G1, driving the G1/S phase transition. Its tumour suppressor activity is due to its ability to induce growth arrest, differentiation or senescence. Although p21 can be stimulated independently of p53, it can also mediate p53 tumour suppressor activity.(263) The function of p21 in relation to p53 is shown in Figure 4. A summary of the studies evaluated demonstrating a significant correlation between p21 expression and response to LCCRT is demonstrated in table 12. Kuremsky *et al* identified four studies where a correlation of p21 was found with clinical outcome.(213,230,264–266)

Spolverato *et al*'s subsequent review only identified one study that identified any correlation between p21 and neo-adjuvant therapy response.(231) Sturm *et al* found expression of p21 levels in pre-treatment biopsies was significantly higher in patients with either complete or partial remission when compared to non-responders.(267)

These studies have conflicting results and although several of the studies demonstrated improvements in disease-free and overall survival, no significant findings were evident on pathological staging. Thus, use of p21 as potential biomarker would require further assessment using prospective trials, but again given the current lack of evidence and conflicting associations in relatively small studies, the role of p21 as a future biomarker also seems doubtful.
Author(s)	Year of	No of	LCCRT regimen (unless	Significant study endpoint	Summary of significant
	publication	patients	otherwise stated)		outcome(s)
Rau et	2003	66	Not stated but delivered in n =	Pathological response with responders	Low p21 expression
al(264)		patients	35 or combined with	classed as those with complete	associated with lack of
			hyperthermic	remission (absence of tumour cells),	response (<i>p</i> < 0.05)
			chemoradiotherapy (n= 31)	partial remission (decrease in depth	
				tumour infiltration or T-stage)	
Charara <i>et</i>	2004	57	45-54Gy + 5-FU + irinotecan	Pathological response classed as	Patients expressing p21
al(265)		patients		complete response (no microscopic	12/30 (40%) had a complete
				residual tumour) or partial response	response vs. 0/10 patients
				(residual but reduced tumour)	with negative expression (<i>p</i> =
					0.011)
Reerink et	2004	34	45-56Gy + 5-FU + leucovorin	Survival	Positive expression of p21
al(266)		patients	+/- intra-operative		correlated significantly with
			radiotherapy 10Gy (n = 11)		worse survival ($p = 0.005$)

Table 12) Table summarising the studies analysed that demonstrated a correlation between p21 analysis and response to LCCRT

Author(s)	Year of publication	No of patients	LCCRT regimen (unless otherwise stated)	Significant study endpoint	Summary of significant outcome(s)
Bertolini <i>et al</i> (230)	2007	53 patients	50Gy +5-FU	Dworak TRG	No correlation identified between p21 and pathological response
				4-year disease-free survival and overall survival	High p21 expression associated with worse disease-free ($p = 0.036$) and overall survival ($p = 0.0006$)
Sturm et al(267)	2006	66 patients	45Gy + 5-fU + leucovorin + (n = 35) or combined with regional hyperthermia (n= 31)	Pathological response with responders classed as those with complete remission (absence of vital tumour cells), partial remission (decrease in depth tumour infiltration or T-stage)	p21 expression was found to be significantly higher in responders in those treated with LCCRT ($p = 0.03$) but not the overall group treated with LCCRT and regional hyperthermia ($p = 0.4$)

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; TRG – tumour regression grade)

1.13.6 Analysis of bax (bcl-2 associated X protein)/bcl-2 (b-cell lymphoma-2)

Both bax and bcl-2 are members of the bcl-2 family, which are responsible for apoptosis. Expression of pro-apoptotic genes can alter with cancer and lead to decrease tumour suppression. Bcl-2 has been identified as a prosurvival protein, whereas bax is pro-apoptotic.(268) The role of bax and bcl-2 in apoptosis is discussed above in section 1.12.1.1. A summary of the studies evaluated demonstrating a significant correlation between bax and bcl-2 expression and response to LCCRT is demonstrated in table 13.

Only one of three studies identified by Kuremsky, demonstrated a significant correlation between bax expression and response to neo-adjuvant chemoradiotherapy.(213,269) Of the 12 studies identified reviewing the role of bcl-2, only Kudrimoti *et al* found a correlation.(270) Spolverato's paper identified two subsequent studies that identified a correlation between bax and bcl-2 and response to neo-adjuvant therapy.(231,252,262)

All of the three studies that identified a significant correlation between bax and response, noted that increased bax expression was associated with greater tumour response. Although there are a limited number of studies, these studies were relatively large and therefore bax may prove to be a potential predictive biomarker and thus warrants further investigation. However, use of bcl-2 as predictive biomarker is unlikely given the lack of studies demonstrating a significant relationship. The two studies identified had contradicting outcomes, however Kudrimoti's finding of increased expression in association with greater tumour response was identified in a study of only 17 participants.(270) **Table 13)** Table summarising the studies analysed that demonstrated a correlation between bax and bcl-2 analysis and response toLCCRT

Author(s)	Year of	No of	LCCRT regimen (unless	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	otherwise stated)		
Chang et al(269)	2005	130 natients	50.4Gy + 5- FU/capecitabine +	Dworak TRG	Bax expression significantly associated with response with 15/28 (54%) natients
ui(20))		putients	leucovorin		undergoing complete response expressing bax
					vs. 30/102 (29%) partial responders (<i>p</i> =
					0.017)
Kudrimoti	2007	17	50.4-59.4Gy (mean	pCR (with responders being	Bcl-2 expression in 3/5 (60%) of responders
et al(270)		patients	55Gy) + 5-FU	classed as those with no	vs. 2/12 (16%) of partial responders
				visible cells in pathological	
				specimen)	
Kikuchi et	2009	60	45Gy + S1 (tegafur-uracil	Dworak TRG	High bax scores correlated with responders
al(252)		patients	+ gimeracil +oteracil) +		(classed as TRG 3 and 4) in both univariate (<i>p</i>
			irinotecan		< 0.05) and multiple logistic regression ($p =$
					0.001) analyses
Huerta <i>et</i>	2010	38	50.4Gy + capecitabine	Response (classified as good	Bax expression found to be higher ($p < 0.001$)
al(262)		patients		being >50% pathological	and bcl-2 expression found to be lower (<i>p</i> <
				response and poor as	0.001) in those defined as good responders
				<50%)	
	Jorouracily I CC	DT Long	ourse chemoradietheran	v nCP complete nathologic	al recoonce TPC tumour regression

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; pCR – complete pathological response; TRG – tumour regression

grade)

1.13.7 Analysis of Survivin (Baculoviral inhibitor of apoptosis repeat-containing 5/BIRC5)

Survivin is the smallest member of the IAP gene family and is involved in the control of mitosis, regulation of apoptosis and the cellular stress response. Its role in apoptosis is discussed in section 1.12.1.1 (Figure 9a). Overexpression has been detected in all varieties of tumour and has been correlated with apoptotic resistance, metastasis, bypass of cell cycle checkpoints, as well as resistance to therapy.(271) A summary of the studies evaluated demonstrating a significant correlation between bax and bcl-2 expression and response to LCCRT is demonstrated in table 14. Franz Rödel and his colleagues have published extensively on the role of survivin in various cancers including rectal cancer. High levels of pretreatment apoptosis have been correlated with Dworak tumour regression after neo-adjuvant therapy.(272) The group subsequently assessed the effect of using short interfering RNA (siRNA), induced down-regulation of survivin mRNA and protein on colorectal cell lines to identify mechanisms of radio-resistance.(273)

Sprenger *et al* evaluated survivin expression within the setting of randomised phase III trials, in both pre-treatment biopsies and their corresponding surgical specimens, from 116 patients with locally advanced rectal cancer (UICC II/III), which underwent neo-adjuvant chemoradiotherapy.(274) Of these patients, 11 (9.5%) achieved complete pathological response and thus no surgical specimen was available to calculate survivin expression. Down-regulation of survivin expression was also seen after neo-adjuvant therapy (p < 0.0001).

Although these two studies demonstrate the potential of survivin as a predictive biomarker, with high levels suggestive of decreased tumour response and inferior outcomes, there is also evidence to support its use as a potential indicator of prognosis.

Krieg *et al*'s relatively recent meta-analysis identified 1934 patients across 15 studies, but looked at all locations of colorectal cancer, but were not able to perform subgroup analysis for rectal cancer specifically.(275) The pooled hazard ratio demonstrated high survivin levels were associated with a decrease in overall survival (p < 0.00001). It is worth noting that only two of the studies evaluated in this meta-analysis focused specifically on rectal cancer and therefore, it was only in these that neo-adjuvant therapy was administered.(276,277) Takasu's study also looked at alteration in survivin expression after neo-adjuvant therapy and although overall expression did not significantly differ in either non-responders or responders, when looking specifically at cytoplasmic and nuclear expression, significant alterations were demonstrated.(277) Despite the relatively low numbers in these studies, further assessment of survivin as a biomarker should be considered.

Author(s)	Year of	No of	LCCRT regimen	Significant study	Summary of significant outcome(s)
	publication	patients	(unless otherwise	endpoint	
			stated)		
Rödel <i>et</i>	2005	59 patients	50.4Gy + 5-FU	Mean apoptotic	Mean apoptotic index for the 21 tumours with high
al(273)				index of pre-	survivin expression (1.3%) vs the 38 tumours with
				treatment biopsies	low expression (2.0%) ($p < 0.0001$)
				5-year incidence	High survivin expression 'significantly related' to
				of local relapse	increased risk of local relapse (26% vs 6%) ($p = 0.05$)
Sprenger et	2011	116 patients	50.4Gy + 5-FU (n = 73)/	Stage of surgical	High levels of pre-therapeutic survivin expression
al(274)			5-FU + oxaliplatin (n =	specimen	correlated with more advanced tumour stage post
			43)		treatment (ypT – p = 0.026; ypUICC - p = 0.005)
				Disease free	Patients with low levels of survivin had increased
				survival	disease free survival rates when compared to those
					with high expression ($p = 0.038$)
Krieg et	2013	1934	Variable – only used in	Prognosis	Pooled hazard ratios of 11 studies (n = 1528) that
al(275)		patients	the two studies that		performed survival analysis demonstrated
		across 15	evaluated rectal ca		correlation between high survivin expression and
		studies			poor prognosis (HR 1.93% CI 1.55-242; <i>p</i> <0.000001;
					$I^2 = 23\%$)
Knutsen <i>et</i>	2004	98 patients	No neo-adjuvant	Survival	In patients that had undergone neo-adjuvant
al(276)			therapy (n = 57)	Recurrence	radiotherapy, survivin expression was not related to
			Radiotherapy (25Gy) in		survival (p = 0.19, local (p = 0.52) or distant (p = 0.41)
			n = 41		

Table 14)Table summarising the studies analysed that demonstrated a correlation between survivin analysis and response to LCCRT

Author(s)	Year of	No of	LCCRT regimen	Significant study	Summary of significant outcome(s)
	publication	patients	(unless otherwise	endpoint	
			stated)		
Takasu <i>et</i>	2012	43	40Gy + S1 (tegafur-	Clinical response	Positive survivin expression in pre-therapeutic
al(277)		patients	uracil + gimeracil		biopsies correlated with lack of response to
			+oteracil)		chemoradiotherapy in 17/22 (77%) vs 9/21(43%)
			chemotherapy		patients with partial response $p = 0.02$)
				Pathological response	Positive survivin expression correlated with non-
				(with non-responders	responders to chemoradiotherapy in 18/24 (75%)
				defined as Dworak TRG	vs $8/19(42\%)$ patients with partial response $p =$
				0-1)	0.01)

(5-FU – 5-Fluorouracil; LCCRT – Long course chemoradiotherapy; pCR – complete pathological response; TRG – tumour regression grade)

1.13.8 Whole genome sequencing

1.13.8.1 Gene expression profiling

Gene expression profiling is a method of identifying the pattern of gene expression at the level of transcription to provide an overall view of cellular function. DNA microarrays have been used to assess the relative activity of previously identified target genes or sequencing methodologies that allow profiling of all active genes.(278) The major advantage over conventional methods of analysis is the ability to assess the expression of thousands of genes simultaneously.(279,280) Gene expression profiling has been used to investigate the genetics of colorectal cancer response to chemoradiotherapy. A summary of the studies evaluated demonstrating a significant correlation between gene-expression profiling and response to LCCRT are demonstrated in table 15a and 15b.

1.13.8.1.1 In-vivo studies

Ghadimi *et al* were the first to report on the analysis of pre-therapeutic biopsies of locally advanced rectal carcinomas for gene expression signatures using microarrays.(281) Biopsies were taken from a subset of thirty patients enrolled in the German Rectal Cancer Trial, a phase III clinical trial. The study assessed a limited subset of patients from the pre-operative arm of the German Rectal Cancer Trial, which the authors acknowledged. However, as a result of the study being conducted within a phase III clinical trial, treatment regimes were standardised.

Watanabe *et al* subsequently published a prospective cohort study of 52 rectal cancer patients undergoing neo-adjuvant radiotherapy without concurrent chemotherapy.(282) Although gene expression profiles identified a list of 33 genes that were differentially expressed at significant levels (p < 0.05) between responders and non-responders, none of these were identified in Ghadimi's study.(281). Five of the genes identified were related to apoptosis: lumican, thrombospondin 2, galectin-1, cyclophlin 40 and glutathione peroxidase 2. Although the authors acknowledged the limitations of the study including the relatively low number of patients used to confirm the validity of their predictive model, they stated that they were attempting to validate these findings using a larger cohort (n > 200), however, this work does not appear to have been subsequently published.(283)

Kim et al's microarray gene expression analysis did not contain any patients deemed to be grade 0, i.e. non-responders and were also not able to identify a set of genes to predict for poor responders (grade 1), which is also of clinical importance in identifying patients who are unlikely to benefit from neo-adjuvant treatment.(284) Of the set of 95 "predictor" genes, only two were noted to be associated with apoptosis: TNF receptor-associated factor 4 and programmed cell death 4. The authors did note that the TS gene was highly expressed in complete responders compared with partial responders. Rimkus et al reported on the gene expression signature of 42 genes to predict neo-adjuvant chemoradiotherapy, five of which are involved in apoptosis: caspase 1, TIAL1, TNF receptor superfamily member 1B, tumour differentially expressed 1 and succinate dehydrogenase complex subunit C.(285) Quantification of the expression of caspase 1 using an independent method was subsequently performed on a set of 21 rectal carcinomas, among which ten were responders and eleven were non-responders. Although caspase 1 was found to be 1.7-fold higher in responders than nonresponders, significance was not reached, which the authors attributed to their small cohort.

Brettingham-Moore *et al* studied pre-treatment biopsies of 51 locally advanced rectal cancers, to compare gene expression in relation to response.(286). Using the predictive genes previously described by above Ghadimi(281), Kim(284) and Rimkus(285), Brettingham-Moore's team tested these on their cohort. Unfortunately these previously identified gene profiles were not shown to be sufficiently sensitive or specific and yielded sensitivities ranging between 21% and 50% and specificities ranging between 30% and 70%. Comparison of the above gene profiles identified that there were no shared genes, however, it was noted that the TNF pathway was a common finding. This discrepancy was felt attributable to several reasons: firstly, definitions of response and resistance to neoadjuvant treatment were assessed according to different measures; a variation in sample numbers; and finally clustering of high dimensional data, where due to the large number genes identified, some of these genes will not be meaningful for a given cluster, and yet others may be correlated.(283)

Although the use of gene expression profiling to predict rectal cancer response to neo-adjuvant therapy is currently limited, it may have potential in the future to aid in identification of responders and non-responders. This method of response prediction has been shown to be effective in identification of patients that will benefit from adjuvant therapy in breast cancer management.(287) The 70-gene prognosis profile is being assessed in a prospective, multicentre (119 institutions in 9 European countries), randomised study, MINDACT (Microarray In Node negative Disease may Avoid ChemoTherapy) with the aim of comparing the gene expression signature with common clinical-pathological criteria in selecting patients for adjuvant chemotherapy in node-negative breast cancer.(288–290) Although initial data has been promising, complete outcome data is expected in 2016.

1.13.8.1.2 In vitro studies

Amundson *et al*'s study tested the sensitivity of 60 cell lines of the National Cancer Institute Anticancer Drug Screen (NCI60) (discussed further in section 1.14.2).(291) They noted that the most strongly upregulated genes in the NCI60 that showed the greatest response were in p53 wild type cell lines with the identification of 25 p53-dependent genes. Eschrich *et al* successfully used a gene expression model to create a predictive tool of response using biopsies taken prior to neo-adjuvant therapy in those with oesophageal (n = 12) and rectal cancers (n = 14).(292) Spitzner *et al*'s study of human colorectal cell lines revealed gene expression levels correlated with sensitivity to chemoradiotherapy.(293) It is worth noting that only two of the cell lines were established using rectal cancers due to limited number

available and thus the remaining ten were colonic in nature, which may explain the discrepancy in these findings, along with the variation in definition of response between this study and the authors' previous work.(281) As seen in the *in vivo* studies, there was minimal overlap in the gene profiles identified, which could be attributed to differences in radiation dose and use of chemotherapy and also due to the multiple cancers represented by both Amundson(291) and Eschrich(292). **Table 15a)** Table summarising the *in-vivo* studies analysed that demonstrated a correlation between gene expression profiling andresponse to LCCRT

Author(s)	Year of	No of	LCCRT regimen	Significant study endpoint	Summary of significant outcome(s)
	publication	patients	(unless		
			otherwise		
			stated)		
Ghadimi et	2005	30	50.4Gy + 5-FU	Response defined as either:	Initial 23 patients used to build predictive model:
al(281)		patients		- decrease in T-stage by at	- 54 differentially expressed genes (<i>p</i> < 0.001)
				least one level or	identified with significance between responders
					and non-responders based on T level downstaging.
					Response prediction in 83% of patients ($p = 0.02$)
				- Dworak TRG 3 & 4	- 5 differentially expressed genes (<i>p</i> < 0.001), but
					probability of expression occurring due to chance, p
					= 0.31. Response prediction not performed.
Watanabe <i>et</i>	2006	52	50.4Gy	Response defined as grade 2	Initial 35 patients used to build predictive model:
al(282)		patients		or 3 according to Japanese	33 genes differentially expressed ($p < 0.05$)
				Classification of Colorectal	between responders and non-responders.
				Carcinoma	Class prediction accurate in 31/35 (88.6%) of
					training group and 14/17 (82.4%) of test cohort

Author(s)	Year of publication	No of patients	LCCRT regimen (unless otherwise stated)	Significant study endpoint	Summary of significant outcome(s)
Kim <i>et al</i> (284)	2007	46 patients	45Gy + 5.4Gy per 3- fraction boost to primary tumour + 5-FU + leucoverin (n = 20)/capecitabine (n = 22)/irinotecan + capecitabine (n = 4)	Dworak TRG	Initial 31 patients used to build predictive model: 261 genes differentially expressed (<i>p</i> < 0.01) between partial response (TRG 1, 2 or 3) and complete response (TRG 4). 'Top-ranked' 95 genes (<i>p</i> = 0.0008) used for predictive model. Class prediction accurate in 26/31 (84%) of optimisation samples and 13/15 (87% of validation samples.
Rimkus et al(285)	2008	43 patients	45Gy + 5-FU	Becker's TRG (modified version of Mandard)(294)	Gene expression profiles generated to identify the 50 probe sets with the lowest <i>p</i> values representing 42 genes differentially expressed between responders (Becker TRG 1) and non- responders (Becker TRG 3). Sensitivity (correct classification of responders) 71% and specificity (correct classification of non-responders) 86%
Brettingham- Moore <i>et al</i>	2011	51 patients	50Gy + 5-FU	Response with responders classed as <10% residual tumour and non-responders as >50% residual tumour.	Sensitivity of 82%, but specificity was only 30%. Using metabolic response to class response, a specificity of 89% was achieved, however this method was unable to detect responders.

Table 15b) Table summarising the *in-vitro* studies analysed that demonstrated a correlation between gene expression profiling andresponse to LCCRT

Author(s)	Year of	No of cell	Radiation	Significant study endpoint	Summary of significant outcome(s)
	publication	lines	dose		
Amundson	2008	60 containing	0, 2, 5, 8 or	Clonogenic survival and gene	22 genes associated with low survival after 2Gy.
et al(291)		9 tumour	16Gy	expression changes	14 genes associated with low survival after 8Gy.
		types (NCI			
		60)			
Eschrich et	2009	48 human cell	2Gy	Radiosensitivity (survival fraction	Ten-gene expression model used to predict
al(292)		lines (from		at 2Gy) and pathological response	response clinically in patients with oesophageal (n
		NCI 60)		(decrease in T-stage by at lease	= 12), rectal (n = 14).
				one level between EUS and	Combined rectal and oesophageal cancer cohorts,
				histopathological response	model able to predict pathological response; mean
					predicted radiosensitivity index in responders
					0.34 vs. 0.48 in non-responders (<i>p</i> = 0.002).
					Subgroup analysis performed in the rectal cancer
					cohort and also found to be significant ($p = 0.03$).

Author(s)	Year of	No of cell	Radiation	Significant	Summary of significant outcome(s)
	publication	lines	dose	study	
				endpoint	
Spitzner <i>et</i>	2010	12 human	2Gy + 5-FU	Surviving	Range of surviving fraction between cell lines of 0.28 and 0.81 with
al(293)		colorectal cell	(3µM)	fraction	5-FU increasing sensitivity in the majority of cell lines.
		lines			Using linear model analysis, 4,796 features identified that
					correlated with sensitivity to chemoradiotherapy across 2,770
					genes with 2,065 features that increased with resistance and 2,731
					that decreased with resistance.

(5-FU – 5-Fluorouracil; NCI – National Cancer Institute)

1.13.8.2 Chromosomal anomalies

Grade *et al* used metaphase comparative genomic hybridisation, to prospectively analyse biopsies taken prior to neo-adjuvant therapy from 42 patients with locally advanced rectal cancer in order to identify genomic imbalances.(295) When comparing responders (downstaged by at least one T level between EUS and histopathology of the surgical specimen) with nonresponders, three different gene band groups were identified that were significantly associated (p < 0.05) with response. It was noted however that the probability of detecting these copy number changes by chance was high (p = 0.21).

Chen *et al* followed this study by using comparative genomic hybridisation to identify chromosomal copy alterations to predict complete pathologic response.(296) The chromosomal regions noted to differ between patients exhibiting a pCR and those that did not contained 473 genes, however, after *p* value correction for multiple testing, only 285 of these were significantly different. Using ingenuity pathway analysis, eight genes in the imbalanced chromosomal regions were identified that were associated with response, diagnosis, prognosis and therapeutic efficacy: ENO2, TPI1, GAPDH, CD4, ING4, CD27, TNF RSFIA and SCG5. Sensitivity and specificity of predicting complete pathologic response using a biomarker model was 76% and 97% respectively.

1.13.9 Future methodologies to validate molecular and clinical biomarkers

Grade *et al* note that almost all the biomarkers that have been identified up to this point have been in the setting of a retrospective study and have not been independently validated as a prospective study using standardised analytical protocols.(283) The TransValid-KFO179/German Rectal Cancer Study Group-Trial, which has been funded by the German Research Foundation, was established to prospectively validate previously identified molecular and clinical biomarkers. This multi-centre study aims to evaluate a panel of markers including TS, epidermal growth factor, vascular endothelial growth factor, as well as potential new biomarkers CD133, XIAP, ERCC1/2 and HER2. The trial has been divided into two parts: TransValid A, a validation study (n = 200) and TransValid B, feasibility study. Findings are yet to be fully published, however, this trial should be able to provide validation.

1.14 Microfluidics and development of cell models

1.14.1 History of Microfluidics

Microfluidics involves the use of a system to manipulate minute quantities of fluid $(10^{-9} - 10^{-18})$ litres) through channels using the principles of laminar flow.(297,298) This allows the miniaturisation of previous techniques and has led to the label, "lab on a chip". There are many advantages of this technology over conventional techniques, with one of the main benefits being the ability to study reactions in a highly efficient way with fine temporal and spatial control. Other benefits identified include: separations and detections being carried out with high resolution and sensitivity, where the technology can be associated with automatic readers to diminish the role of human error; use of enclosed devices, preventing the evaporation that occurs from open wells; relative low cost to manufacture the devices; short times for analysis; and integration with other technologies to perform multiple functions consecutively.(297–299) In addition, due to the small size of the device and thus requirement of only small quantities of samples and reagents, there is a relatively low associated cost due to the small volumes of reagents required.

The concept of microfluidics development was based on contributions from techniques used within a variety of other environments and modified to create the systems that are now currently used.(298) Micro-analytical methods were initially used in the application of gas phase chromatography, which circulated gas through micro-channels etched in silicon and included use of miniaturised electromagnetic detection and thermal detection all contained on the same chip.(300) Subsequently, high-pressure chromatography and capillary electrophoresis also used this miniaturised

technology. Further development of these systems continued with the advent of optical lasers, which allowed the use of small sample quantities, without compromising the associated high sensitivity and resolution. In the 1990's, the US Department of Defence funded programmes to develop microfluidic systems for use in austere environments, including the detection of biochemical weaponry.(301) The field of molecular biology required analytical methods that were faster and had a higher sensitivity and resolution than previous methods, whilst being potentially portable. Within the field of molecular biology, there was an increasing demand for a technology that offered a solution to the relative low throughput of previous technologies and this led to its use within immunoassays, separation of proteins and DNA, sorting and manipulation of cells, as well as examination of cell biology using laminar flow.(302) Finally, micro-electronics technology was applied to create microfluidic devices.(303) Parallels that have been drawn between the two technologies have highlighted their similar manufacturing process and their miniature scale.

Although the initial devices used were made up of silicon or glass, as previously used within silicon micro-electronics and in microelectromechanical systems, this has been superseded by the use of plastic polymers, particularly due the lack of gas permeability of both glass and silicon, which was of particular concern when working with live mammalian cells.(298) Plastics were also found to be advantageous for this purpose for several reasons including: being relatively economical, relative flexibility for fabrication of devices, and opacity to visible and ultraviolet light, thus permitting use with conventional methods of detection.

This has led to the use of polymers such as polydimethylsiloxane (PDMS), an optically transparent soft elastomer.(304) PDMS has many other properties that have made it advantageous, particularly within the field of biological studies, due to its excellent permeability to gases, allowing the gaseous exchange of oxygen (O₂) and carbon dioxide (CO₂), in addition to also being non-toxic to cells.(302) Although PDMS is inexpensive, flexible and optically transparent, its major advantage over glass and silicon is the ease in which it

can be bonded to other surfaces in order to create microfluidic devices. The devices are manufactured using computer-aided design (CAD) programs in conjunction with photolithographic etching techniques.

Microfluidic devices have been manufactured in variety of different ways to accommodate the multitude of specialties in which they can be used. These include the potential to screen large number of conditions for protein crystallisation; separations coupled to mass spectroscopy; high-throughput screening in drug development; bioanalyses; examination and manipulation of samples consisting of a single cell or a single molecule; and synthesis of 18F-labelled organic compounds for positron emission tomography (PET).(298)

Microfluidic technology first became commercially available in the production of inkjet printer heads in the 1980s, but has since been incorporated into the manufacture of biomedical devices.(305,306) In 1999, Agilent Technologies released the 2100 Bioanalyzer, the first commercial microfluidics device for use with biological samples to analyse DNA, RNA, proteins and cells as an alternative to electrophoresis and flow cytometry.(307) The advantages cited include speed of analysis with results delivered within 40 minutes; flexibility; sensitivity (down to concentrations of picogram per microliter) and reproducibility due to standardisation and automation. Additional benefits include the system only requiring minimal sample consumptions of 1-4 μ L, as well as the relative small size (290 x 162 x 412mm) of devices.

The role of microfluidics has also become clinically, as well as commercially apparent and development of the technology in conjunction with microelectronics has led to the production of devices that can perform diagnoses at the bedside, often referred to as point of care (POC) devices (Figure 12).(308) One example of this technology is the Alere Triage® System, a microfluidic chip, which used in conjunction with a miniaturised computer analyser can analyse a drop of blood for the presence of proteins released by damaged cardiac muscle to identify if patients have suffered damage to cardiac muscle and thus diagnose heart attacks (Figure 13).(309,310) This

technology is advantageous over traditional methods: due to its size, portability of the system and results that can be rapidly obtained. Therefore a diagnosis can be obtained within 15 minutes, which is far quicker than conventional methods, and eliminates the transport of samples prior to analysis which can take up to several hours. This technology has been applied to many other contexts within medical field including CD4+ T-cells for monitoring HIV/AIDS, monitoring drug metabolism including warfarin, and monitoring of prostate specific antigen (PSA) in the management of prostate cancer.(310)



Figure 12) A diagrammatic example of a Micro Total Analysis System used as a POC device for diagnosis of diseases such as HIV as adapted from Chin *et al*(311). A preloaded sequence of reagents pass over four successive detection zones, before leaving the chip to a syringe used to create a vacuum for fluid actuation.



Figure 13) The Alere Triage[®] MeterPro, which is a example of point of care device using microfluidic technology that allows rapid testing of body fluids, including blood to identify critical conditions including pulmonary embolus and myocardial infarction. Image obtained from Alere.com.(312) Other devices manufactured by the company have also been used for rapid identification of viruses and bacteria.

Microfluidics has also been used by a variety of diagnostic medical apparatus in the early diagnosis of diseases, such as the use of cell-specific surface markers to identify cancer.(313) Du et al utilised a PDMS microfluidic device to identify human cervical cells using a capture antibody.(314) Harvested cervical cancer cells were preferentially captured using α 6-integrin cell surface receptors over normal human glandular epithelial and cervical stromal cells. This paper gives rise to the potential for detection of cancerous cells using other tumour markers. Nagath *et al* applied microfluidic technology using antibody coated pillars to identify circulating tumour cells in the blood of patients suffering from epithelial cell tumours (lung, prostate, pancreatic, breast and colon cancer) to identify metastases.(315) Of the 116 patients samples analysed, 115 (99%) were successfully identified as containing circulating tumour cells, with a sensitivity of 99.1% and specificity of 100%. Weigum *et al* assessed the role of a microfluidic device to detect overexpression of EGFR, a biomarker of oral cancer, using three human oral tumour derived cell lines.(316) EGFR

was used a as a proof of principle biomarker, with a significant increase in expression in relation to control cells. This assay was completed in less than ten minutes, further corroboration of the potential expeditiousness of the technique.

1.14.2 Development of models to maintain cells ex-vivo

1.14.2.1 Cell lines

Conventionally tumour behaviour has been investigated by using immortalised cell lines to create a simple model of the tumour, however the main disadvantages of these systems is that they do not represent the tumour's true complexity.(317) By the 1950's animal cell culture had become routine practice, but in 1951, HeLa, the first human cell culture was developed from a patient with cervical adenocarcinoma.(318) In the 1980's, the United States National Cancer Institute (NCI) developed the NCI60 programme to replace the use of animal models, due to their relative expense, the lengthy procedure time, concerns over accuracy, as well as ethical considerations.(319,320) In 1990, they launched a protocol to ensure that potential anti-cancer drugs could be tested in a high-throughput manner on 60 different human cell lines, which represented nine different cancers, including that of the colon with seven cell lines. The intention of the screen was to identify compounds with growth-inhibitory or toxic effects on specific tumours, however patterns of drug sensitivity/resistance were also identified. The COMPARE algorithm as created by Paull *et al*, has been used to assess response of cell lines to these compounds and has identified that compounds with similar cell line sensitivity profiles tend to have similar mechanisms of action.(321) Johnson et al performed an analysis of 39 compounds tested both *in vitro* using the NCI's Developmental Therapeutics Programme and also in phase II studies using hollow fibre models (as described in section 1.14.2.4).(322) In vitro histology was demonstrated to significantly correlate with *in vivo* activity using breast, lung, ovarian, CNS and melanoma cell lines (p < 0.003), however with colonic cell lines no correlation in activity was seen (p = 0.670).

The programme has been designed to screen up to 3000 small molecules per year. The screen is now used predominantly to assess various compounds across the cell lines to create algorithms based on pattern recognition. These algorithms are then used to assess submitted agents to identify potential mechanisms of action, determine unique response patterns and identify that the tested compound is not similar to any of the standard prototype compounds in the NCI database.

Subsequently the Japanese Foundation for Cancer Research created the JFCR-39, a panel of 39 human tumour derived cell lines, thirty of which are part of NCI60 in addition to six gastric cancer lines and three breast cancer cell lines.(323) Again JFCR-39 functions as platform for drug discovery, but has also been used to identify a protein biomarker, prothymosin- α on colon cancer cells.(324) The expression of prothymosin- α was found to be higher in colon cancer cells than in comparative normal colonic cells. Since the creation of these two databases, further cell line databases have been created, with one of the largest being the Sanger Cancer Cell Line Project, which contains over 1000 cell lines.(325)

There have been several limitations cited with the use of cell lines, in that they are not truly representative of *in vivo* tumours.(317) Only cell autonomous sensitivities can be assessed using cell lines, whereas therapies may also alter tumour cell interaction with its environment (e.g. drugs that inhibit angiogenesis). Screening using this platform requires rapidly dividing cell lines to detect cytostatic or cytotoxic activity and thus slow growing tumour cell lines may not produce desired measurable effects. Culture mediums and the use of foetal bovine serum (FBS) are only a substitute for growth *in vivo*. Oxygen levels delivered to the cell culture are also unlikely to be a true representation of that *in vivo* and thus compounds that rely on the formation of reactive oxygen species or hypoxia-dependent pathways may be affected. The range of mutational changes present in tumour cells may not be represented by cell lines and therefore not demonstrate variability in drug sensitivity *in vivo*. Finally some tumour types are difficult to maintain *in vitro* and tumour cells from various stages

and cancer subtypes may not propagate. Consequently these tumours are not truly represented in the cell line resulting in possible loss of phenotypic properties. In a bid to create a model that better mimics the *in vivo* environment, work has been carried out to develop three-dimensional models, including: multi-layer cell systems; matrix-embedded 3D cultures; hollow fibre assays; multicellular tumour spheroids and ex vivo tumour cultures.

1.14.2.2 Multi-layer cell systems

Cowan et al first introduced a multilayer cell system, whereby the Chinese hamster cell line, V79-171b was cultured on collagen coated Teflon® membranes.(326) The authors assessed the diffusion of various substances across the multicellular membrane to determine the kinetics across it and create a reliable method of studying the extravascular transport of various chemotherapeutic agents. Following this, Minchinton et al developed threedimensional murine cell cultures of up to twenty cell diameters thickness.(327) Cervical squamous cell carcinoma cultures were grown on a semi-permeable membrane, suspended in stirred media to form a 'thick mat of cells'. These multi-layer cell cultures were then irradiated to assess radiosensitivity in comparison to cells in suspension that were either, deprived of or in the presence of oxygen. It was demonstrated that cell survival curve characteristics of the multi-layer cell cultures were of a mixed population of both oxic and hypoxic cells, similar to that found *in vivo*. The multilayer cultures were also exposed to tirapazamine, a cytotoxic agent that is only activated at low oxygen levels as seen in hypoxic areas of tumours. This experiment confirmed the hypoxic nature of deeper cells within the multilayer system. These models exhibited many of the characteristics of solid tumours *in vivo*, including acting as a barrier to the diffusion of small molecules, necrosis in areas distal to the media, and areas of hypoxia. Human colonic cell lines including that of the adenocarcinoma cell lines, Caco-2, HT29 and SW60 have also been optimised to create multilayer cell cultures that have been used to aid the screening of drugs.(328-

330) Examples of methods of multilayer cell formation are demonstrated in figure 14.

Although these models are able to mimic heterogeneity within the tumour, they lack an essential component of tumour biology, the extra-cellular matrix and therefore tumour behaviour cannot be fully assessed. Tumour interaction with blood vessels including the blood vessel barrier *in vivo* also cannot be replicated.(331)



Figure 14) Diagrammatic representation of two methods of multilayer cell culture formation as adapted from Haraguchi *et al.*(332) Initially cells are cultured on a temperature-responsive dish until confluence is achieved. These confluent cells can be collected as an intact sheet by reducing the temperature. The cell sheet can then be manipulated by either **A)** simple pipetting method to layer cell sheets or **B)** using a hydrogel-coated plunger-like method, where the cell sheet adheres to the hydrogel surface to stack cell sheets.

1.14.2.3 Matrix-embedded three-dimensional cultures

Kalus *et al* were the first to create a matrix-embedded culture using the explants of 160 human tumours on a matrix of fibrin foam and maintained them for up to 21 days.(333) The explants were demonstrated to maintain their original morphological architecture with the development of similar structures in the outgrowing tumour cells. Subsequently, Yang *et al* further developed three-dimensional cultures using collagen cells to act as a matrix for murine mammary tumours.(334) These cultures led to the formation of duct-like outgrowths resembling mammary tumours *in vivo*. These duct-like projections were transplanted *in vivo* to the gland free mammary fat pads of female mice and at four weeks, palpable tumours were present. The histological characteristics of these excised tumours were demonstrated to resemble the original donor tumours.

A large proportion of work using three-dimensional cell cultures has been performed using breast cell lines, including the work of Bissell and colleagues.(335,336) They noted morphological and biochemical differences between cells grown as two-dimensional and three-dimensional structures. The three dimensional models used cells cultured on a laminin-rich extracellular matrix and were considered to be a more representative model of *in vivo* tissue.

The advantages of three-dimensional cultures over their two-dimensional counterparts are numerous and include mechanical support and signalling mediated through the matrix.(337) Currently, there are two principal methods of producing three-dimensional cultures: use of synthetic polymers or biological products such as collagens or elastins to act as a matrix for the cell cultures to embed (Figure 15).(337) Several advantages have been cited regarding the use of synthetic versus biological produced matrices, including the ability to construct synthetic scaffolds uniformly, whereas variability may be present in the repeated production of a biological matrix. Synthetic scaffolds can also be produced in large quantities unlike biological structures, which are limited by the requirement of living organisms from which to obtain biological material. Synthetic scaffolds can be customised to respond accordingly to environmental cues, such as the presence of

antibodies or enzymes. Synthetic scaffolds are also believed to be more representative of *in vivo* matrices, as cells implanted into the synthetic scaffold dictate the overall characteristics, whereas biological scaffolds retain the characteristics of their *in vivo* counterpart. An advantage however of biological matrices is that they can naturally replicate the diverse extracellular matrices present *in vivo*, whereas different synthetic scaffolds must be constructed for each purpose.



Cell with integrins and other surface receptors

- Growth factors
- Integrin-binding sites

Figure 15) Diagrammatic representation of two types of three-dimensional extracellular matrices: **A)** synthetic polymers that permit cellular viability and allow cells to remodel their surrounding environment and **B)** biological polymers that permit cell signalling cascades, which direct cell behaviour by binding with cell surface receptors. Adapted from Tibitt and Anseth. (338)

1.14.2.4 Hollow fibre models

The Hollow Fibre assay was developed consequent to the NCI60 programme, where a replacement was required for the costly, time consuming xenograft models used to previously validate the initial screen.(339,340) This *in vivo* assay involved initially growing cells within polyvinylidene fluoride (PVDF) hollow fibres with a 500kDa molecular weight cut off. The hollow fibres were subsequently placed within the peritoneal and subcutaneous compartments of mice, allowing assessment of response of the tested compound to enter the compartment. Tumour cells implanted into the hollow fibres were cultured for 24-48 hours *in vitro* and then implanted into athymic (nude) mice. The mice were allowed to recover for three to four days and then most commonly, the compound being tested was administered for four days, after which the hollow fibres were removed (Figure 16).

Analysis of the activity of compounds tested using hollow fibre models and *in vitro* (NCI60) was performed and a highly significant correlation was observed in sensitivity.(322) Anti-neoplastic drugs shown to be effective in the hollow-fibre models were also demonstrated to also be effective in xenograft models.(339)

The system has been used as a pre-screening tool before further testing and also to evaluate novel chemotherapeutics in colorectal cancer.(341,342) The approach has the benefit of being able to use a variety of cell lines that do not form tumours in animals and mimic tumour heterogeneity, in addition to being able perform further *in vivo* studies with the cells cultured in biocompatible fibres and transplanting them into mice. (331) Despite these benefits, several limitations have been identified. A standard panel of only twelve human cancer lines is currently used in the hollow fibre assay therefore limiting its use.(339,343) The methodology used by the NCI required implantation into athymic mice, which is relatively costly and time consuming; however work carried out by Shnyder et al successfully demonstrated that the hollow fibre assay could be carried out using immunocompetent mice with similar cell growth and response to administered therapy.(344) Although the hollow fibre is semi-permeable, it does create an artificial barrier between the cell lines and the surrounding cells and the growth of the cell line is also restricted by the fibre walls, inhibiting the migration of large molecules such as antibodies.(331,345) This restriction in growth necessitates experiments to be performed at a time when cell growth is greatest.



Figure 16) Simplified diagram to illustrate the use of hollow fibre models to as a three-dimensional cell culture to assess compounds adapted from Sharma *et al.*(317) Cells are initially flushed into the semi-permeable PVDF fibres and then heat-sealed. After culturing the cells, the fibres are implanted into the mice. The mice are allowed to recover for three to four days before receiving treatments.

1.14.2.5 *Multicellular tumour spheroids*

Multicellular spheroids were first established for use in amphibian development, but subsequently were adapted by Sutherland *et al* for use in cancer research, where they demonstrated that the morphology and behaviour of spheroids resembled that of solid tumours.(346) The platform was subsequently developed for use as a model to assess response to chemotherapy, immunotherapy and radiation studies, as well as other potential treatments.

Spheroids consist of three-dimensional cellular aggregates, which can also secrete extracellular matrix to thus allow greater cell to cell interaction.(347) Various techniques have been described of how to create spheroids. Initial methods described the 'hanging drop' technique, where there is spontaneous cell aggregation at the bottom of a drop after inversion of a plate containing a cell suspension, which was first described by Lewis and Lewis in 1924.(348) Although this technique has been modified, it still essentially involves the same basic principles.(349) Other techniques described to create spheroids include: mechanical methods that prevent attachment of cells to containing vessel e.g. spinner flasks(346); the liquid overlay technique, where cells are cultured on a non-adherent substrate(350); and mechanical methods that promote cellular aggregation e.g. centrifugation(351) (Figure 17). By seeding tumour cells singly or in conjunction with other cell types, homotypic or heterotypic spheroids can be created.(317)

Luca *et al* studied the impact of extra-cellular matrix on phenotype, gene expression and EGFR signalling using colorectal cell lines.(352) Seven colorectal cell lines, SW-480, HT-29, DLD-1, LOVO, CACO-2, COLO-205 and COLO-206F, were cultured upon a laminin-rich extracellular matrix. The authors identified wild-type K-ras colorectal cancer cells induced to form spheroids, exhibited decreased expression of EGFR in comparison to twodimensional monolayers. Indovina et al used MG-63 human osteosarcoma cells to create monolayer cultures and spheroids, subjecting them both to 5Gy ionising radiation. (353) Their study identified cell death within the monolayers was caspase-independent, whereas that of spheroids involved caspases with increases in Bax and survivin. This would suggest that spheroids are able to more closely mimic in vivo response. This is likely due to the intercellular adhesion present, which is a regulator of apoptosis.(354) Several authors have discussed the radiobiological response of spheroids in that its response resembles that *in vivo* and have been used to assess radiotherapy protocols. (355–357) These findings demonstrate that spheroids could also potentially be used as a predictive tool of radiotherapy response in vivo.

Multicellular spheroids have been reported to be advantageous in multiple respects to monolayer models, as they provide a better physiological platform for interrogation.(356,358–362) Spheroids exhibit a better organisation of cells, with greater cell-to-cell adhesion, including tight

junctions and thus more closely resemble their *in vivo* counterparts. Due to the multicellular arrangement and extracellular matrix deposition, spheroids can better represent the delivery of chemicals, nutrients and other factors within tumours. Diffusion gradients to oxygen, nutrients, metabolites, as well as other chemicals exist within the three-dimensional structure of the spheroid, which more closely emulate the microenvironment of the tumour represented. Within large spheroids, areas of central necrosis can develop, with a surrounding layer of quiescent viable cells and further layer of proliferative cells. Radiation-resistant hypoxic regions present in the centre of solid tumours can result due to poor vascular supply and relatively poor oxygenation. This hypoxic core region may be indicative of response to chemoradiotherapy. Spheroids have been demonstrated to exhibit the same responses as tumours with necrosis and radiation-resistant hypoxic cores. Finally, multiple cell types can be used to create co-culture spheroids, which can more accurately represent the intracellular signalling demonstrated in vivo.

However, there have also been limitations attributed to the use of multicellular tumour spheroids.(317,363) Although spheroids aim to recreate the biological complexity of tumours in vivo, this is only be partially replicated as many cells including stromal and endothelial cells that support development are absent and therefore responses may altered. However with the development of culturing using multiple cells, this may be eliminated in the future. In addition, not all tumour cell lines can be successfully grown into spheroid cultures and Friedrich *et al* demonstrated that only 26 of the 60 cell lines of the NCI60 panel could be established as spheroids.(364)



Figure 17) Diagrammatic representation of multicellular tumour formation as adapted from Sharma *et al.*(317) Single cell suspensions of tumour cells are seeded to ensure cell to cell aggregation, but prevent attachment to the containing body by several different methods including: mechanical methods that prevent attachment e.g. spinner flasks, mechanical methods that promote aggregation e.g. centrifugal compression into a cell pellet, coating tissue culture surfaces with non-adhesive surfaces e.g. Matrigel[®], a solubilised basement membrane preparation or using the hanging drop method. The tumour cells can be seeded singly or mixed with other cells to form homotypic or heterotypic spheroids.

1.14.3 The role of microfluidic devices in maintaining ex-vivo tissue biopsies

Due to previous *in vitro* models being a relatively poor representative model for disease *in vivo*, a new methodology was sought that would act as a better model. Concerns have been voiced with regards to conventional culture techniques, including the supply of nutrients and growth factors in batches, in addition to the build up of waste products, which cannot be excreted, as they *are in vivo*.(365–367) Normal physiological activity within capillaries involves the exchange of metabolites and gases, as well as the excretion of waste products, due to diffusion.(368) It was noted that microfluidics offered similar characteristics of flow and spatial parameters to that seen *in vivo* and therefore that this technology could be used to continuously perfuse tissue.(369,370)

Several authors have previously demonstrated the role of microfluidic technology in the maintenance of both monolayer and three dimensional cell cultures including multicellular spheroids.(371–373) Although there have been several studies maintaining colorectal spheroids within microfluidic devices, only one study has been published on the use of colorectal tissue biopsies to date.(374–376)

Blake *et al* were the first group to successfully maintain *ex vivo* tissue within a microfluidic device using medullary cerebral tissue extracted from rats.(377) Their study involved the use of a PDMS device to perfuse the neonatal rat medullary brain slices, with evidence of tissue viability for up to three hours, to produce respiratory-related motor output. Subsequently, Webster et al successfully maintained human colorectal tissue biopsies for periods of greater than 50 hours within a fabricated microfluidic chip.(376) Functionality was demonstrated as evidenced by alterations in levels of VEGF in the perfusing medium, with VEGF secretion in the tumour biopsies when the environment was made hypoxic. This was not identified in the normal tissue biopsies.

Hattersley *et al* further described this pioneering microfluidic system, in which a biomimetic environment was created to maintain rat liver tissue samples for up to 70 hours.(378) The liver sections were immediately cryopreserved on collection and thawed prior to use within the microfluidic device. Photolithographic and wet-etching techniques were used to create a two-layer glass microfluidic device within which the tissue was placed to allow interrogation. Micro-channels etched into the device were used to continuously to perfuse the tissue with supplemented Williams Media E

and also permit collection of effluent for analysis of metabolites. Despite initial cryopreservation of tissue, it was shown to retain morphology, viability and functionality. Tissue morphology was retained after incubation within the microfluidic device for 71 hours as demonstrated using haemotoxylin and eosin (H&E) staining. Further validation of cell viability was then performed using LavaCell[™], which was confirmed at up to 53 hours. Hepatocyte functionality was also assessed using ELISA to determine that albumin and urea, both products produced within the liver continued up to 70 hours. Although a relatively small study, the work provided a platform upon which a pseudo *in vivo* environment could be created for further interrogation of tissue.

Based on this work, the group successfully used their microfluidic device to maintain biopsies taken from head and neck malignant tumours.(379,380) Fresh biopsies of primary head and neck squamous cell carcinomas (HNSCCs) or metastatic lymph nodes were perfused with media for up to eight days within the microfluidic devices. Experiments were also carried out to assess the effect of snap freezing. Lactate dehydrogenase (LDH) was used as a marker of cell death to analyse collected effluent and was seen to be initially high upon placement within the microfluidic device, but decreased to minimally detectable levels after approximately 12 hours. This was hypothesised to be due to the initial trauma of tissue preparation and stabilisation within the microfluidic device. This pattern was also replicated with the use of frozen specimens. A lysis buffer was infused through the device after 70 hours, which led to a marked rise in LDH levels for five hours due to the rupture of cell membranes. H&E staining confirmed preservation of tissue architecture after maintenance within the devices for 72 hours with evidence of intact nuclei, minimal losses in cell cohesion and specifically no evidence of central necrosis, which supported the ability of diffusion of nutrients and waste products through the biopsy. This platform was then used to interrogate these biopsies with the chemotherapeutic agents, 5-FU and cisplatin, drugs used in the management of HNSCCs. Head and neck biopsies from 23 patients with histologically confirmed SCCs undergoing surgery, were used across a total of 107

microfluidic devices to assess the ability of the platform to mimic the *in vivo* environment. The addition of these chemotherapeutics led to an increase in levels of LDH when compared to untreated biopsies. Cytochrome c, a mitochondrial protein that is released in apoptosis unlike necrosis, was also assessed. Levels were found to be initially low with an increase two to three days after commencement of the chemotherapy agent, before a steady decline. This pattern of release would suggest an apoptotic mechanism of cell death. This innovative work demonstrated the potential of creating a predictive tool, so that individual patients can be treated based on their preclinical outcomes.

Based on these studies, Carr *et al* further adapted this pseudo *in vivo* model to investigate irradiation of HNSCC biopsies with an overall aim of predicting patient response prior to clinical radiotherapy.(381) Murine liver tissue was used to aid optimisation of the model. Each individual biopsy was irradiated within a linear accelerator by housing the microfluidic device within a Perspex phantom to allow accurate and uniform radiation dose delivery. During initial optimisation, tissue was maintained for up to 333 hours after placement into the microfluidic device, with confirmation of viability by assessment of LDH increase after addition of a lysis agent. Upon irradiation of the liver tissue with a single 20Gy fraction, significant increases in LDH were identified in the two-hour period after irradiation. However, significant LDH increases were only identified in SCC biopsies with high doses of irradiation (40Gy). Given this lack of quantifiable response with more clinically relevant doses (≤ 10 Gy), cytochrome c release was also assessed, but again no demonstrable significant increase was observed in the biopsies treated with single doses of 5Gy or 10Gy. Apoptotic indices as calculated by identification of caspase-3-cleaved cytokeratin using M30 were significantly higher than that of the non-irradiated group at all doses (p = 0.006), with a dose-dependent relationship observed. A fractionated course of radiotherapy (5 x 2Gy) was also carried out with a significant increase in apoptotic index also observed between the nonirradiated and irradiated tissue. Although a proof of concept study, tissue was maintained for up to 15 days, demonstrating the potential of the
model for further investigation as a surrogate for the *in vivo* environment and allowing sufficient time for assessment of response to therapeutic measures such as irradiation. Further correlation with clinical response in a suitably powered study may be able to create a predictive tool of response.

1.15 Methodological approaches to assess apoptosis

Based on the work of Webster, Hattersley and Carr, it was decided to interrogate rectal cancer tissue biopsies using the previously used fabricated microfluidic devices.(376,378,380–382) Although Webster had previously investigated the viability of human colorectal biopsies, within this microfluidic device, it was decided that these experiments should be repeated with both cryopreserved, in addition to fresh biopsies as per the method used by Hattersley's group. Murine colorectal tissue was chosen for initial optimisation work to ensure that the colorectal biopsies were preferably reserved for interrogation with radiation. LDH was chosen as the first biomarker to be assessed in the effluent with H&E staining of the tissue within the devices, due to previous work conducted with SCCs. LDH was chosen to predominately assess cell death to determine cell viability. Based on Carr's irradiation work, a similar methodology was chosen with which to interrogate the colorectal tissue.

In a similar manner to the above studies, both the incubated tissue biopsies and the perfusing medium were chosen for analysis to identify potential markers of response. The initial panel of assays chosen to assess response of the tissue to irradiation included M30 CytoDeath[™] to identify caspase cleaved K18 fragments and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL). Both of these assays have been successfully used by Carr *et al* to assess SCC response to irradiation (use of TUNEL not published). TUNEL and M30 CytoDeath[™] identify different stages of apoptosis, with nick-end labelling occurring after cleavage of cytokeratin and therefore by using a panel of markers, the marker of greatest sensitivity for prediction of response could be identified.(383)

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Based on previous work conducted by Professor Thomas-Oates (Mass Spectrometry Centre of Excellence, University of York) to determine alterations in metabolomic profiles, this technique was also chosen to identify if these alterations correlated with clinical response.(384)

1.16 Study aim

The aim of the study was two-fold. It was firstly to successfully maintain rectal cancer biopsies within a microfluidic device; and subsequently, to interrogate this *ex vivo* rectal cancer tissue with radiation and measure changes in morphology and induction of cell death through apoptosis. I chose to focus on a panel of biomarkers of cell death and apoptosis that had been previously investigated using a similar microfluidic device: LDH, M30 Neo-epitope, TUNEL and in addition metabolomics analysis.(378–382) The ultimate long-term objective however, was to create a method of predicting clinical response of locally advanced rectal cancer to neo-adjuvant chemoradiotherapy and therefore ensure that patients can receive personalised therapy based on these results.

SECTION 2: Materials & Methods

2.1 Murine tissue collection

Colorectal tissue for initial optimisation work was obtained from male Wistar rats (*B&K Universal Ltd, Hull, UK*) killed under a Schedule 1 procedure after intraperitoneal anaesthesia (10mg/kg of 10mM sodium thiopentone) at the University of Hull animal house.

2.2 Ethical approval to obtain human rectal cancer tissue biopsies

Ethical approval was gained from the South Yorkshire Research Ethics Committee (REC) (Reference 11/YH/0364) to obtain rectal biopsies from patients diagnosed with rectal cancer and to also collect patient demographic data, tumour characteristics and treatment administered (17 October 2011). Further details can be found within the research protocol (Appendix 2) and the data collection forms (Appendix 3). Approval was also obtained from Hull & East Yorkshire (HEY) NHS Trust Research & Development (R&D) Department (Reference R1237) (10 November 2011).

2.2.1 Anticipated number of patients in study

It was initially anticipated that fifty patients would be recruited to the study over a two-year time period that would undergo long course chemoradiotherapy. This figure was based on the Colorectal department at Castle Hill Hospital treating approximately 100 patients with rectal cancer per year and estimating that at least 60% of patients would agree to study participation. However, it was discovered that not all of these patients presented through the two week wait clinics and hence an amendment was submitted to the REC. The protocol was amended to also enable collection of rectal cancer tissue at the time of surgery in patients that had not undergone neo-adjuvant therapy to increase the potential number of patient samples available for initial optimisation. Ethical approval was again obtained from the REC (30 August 2012) and favourable opinion obtained from the HEY R&D department for this amendment (10 September 2012).

2.3 Patient recruitment

Patients were identified and recruited from the outpatient clinics of Hull & East Yorkshire Hospitals Department of Colorectal Surgery.

These patients were seen in the outpatient clinics of Hull and East Yorkshire Hospitals NHS trust as per the "Two Week Standard". At this appointment, patients underwent a flexible sigmoidoscopy, as per the Portsmouth model previously described by Flashman *et al.*(385) If a rectal lesion suspicious of cancer was identified, the patient was booked for a colonoscopy to further evaluate the remainder of the large bowel and the rectal lesion. As part of their clinical staging, a contrast enhanced Computed Tomography (CT) scan of the thorax and a Magnetic Resonance (MRI) scan of the abdomen and pelvis were also performed, as per NICE guidance.(116) Any patient presenting to the Colorectal Department with a presumed

diagnosis of rectal cancer that required full colonoscopic examination was asked if they would like to participate in this trial and if so were given a trial information pack to read in the interim between initial presentation and attendance for colonoscopy (Patient information sheet (PIS) - Appendix 4). This interval was usually about one week, but patients were provided at least 24 hours.

On the day of colonoscopy, a member of the research team formally approached these patients and consent was obtained if they agreed to participate in the study (Consent form - Appendix 5). Patients were not offered any remuneration for trial participation.

2.3.1 Inclusion criteria

Any patient presenting to Castle Hill Hospital with a presumed diagnosis of rectal cancer requiring a full colonoscopy, biopsy, pelvic MRI and thoracic CT was considered for inclusion within the study.

2.3.2 Exclusion criteria

Pregnant women, patients unable to consent to participation, patients with the presence of a significant coagulopathy and patients that had undergone previous pelvic radiotherapy were excluded from the study.

2.4 Reagents

PAA Laboratories, Little Chalfont, UK, supplied all reagents unless otherwise stated. All supplying companies are listed in Appendix 6.

2.5 Supplemented Dulbecco Modified Eagle Medium (DMEM)

The obtained tissue was maintained in a supplemented DMEM solution as previously shown to be successful in maintaining colorectal tissue biopsy cultures by Webster *et al.*(376)

To 500ml of Dulbecco's Eagle Medium: High Glucose (4.5g/l) without L-Glutamine the following reagents were added:

- Foetal Bovine Serum (FBS) (50ml) (*Biosera, Uckfield, UK*) (Final concentration 10% (v/v))
- Final concentration of Penicillin 0.1U/ml/Streptomycin 0.1mg/ml
- L-Glutamine 200mM (5ml) (Final concentration 2mM)
- Minimal essential medium (MEM) Non Essential Amino Acids (5ml) (Final concentration 1% (v/v))
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Buffer Solution 1M (15ml) (Final concentration 30mM)

2.6 Rectal cancer biopsy sample collection

Tissue collection was undertaken according to the rules and guidelines set out by the Human Tissue Act 2004.(386) Each sample was labelled using an individualised trial number.

During the colonoscopy, the rectal lesion was evaluated and routine histological specimens were obtained by brushing and endoscopic forceps

biopsy. For those patients who had agreed to participate in this trial, a further five biopsies of the rectal lesion, of approximately 2mm diameter, were taken for this research study.

The research specimens were handled in two different ways at this point to either, process the tissue samples immediately, or snap-freeze for later analysis (as previously shown to be viable by Sylvester *et al*)(382):

- Fresh: The samples were transferred into a 50ml polypropylene conical centrifuge tube containing at least 20ml of supplemented Dulbecco's Modified Eagle Medium (DMEM), ensuring that the biopsies were submerged fully (composition of DMEM as described in section 2.4). This was maintained at 4°C and transported to the laboratory and used for immediate analysis.
- 2) Snap frozen: The samples were immediately placed in cryovials, which were then immersed in liquid nitrogen (-196°C). The samples were subsequently transferred to an ultra-low temperature scientific freezer (-80°C). When required, the sample was removed from the freezer and thawed at room temperature for 15 minutes and analysed in the same manner as fresh samples.

2.7 Microfluidics

2.7.1 Microfluidic device

The microfluidic devices were constructed using two layers of glass: the upper layer of 3mm thickness and the lower layer of 1mm. A channel network was created in the lower layer using photolithographic and wetetching techniques as first developed by Broadwell *et al* at the University of Hull.(387) Thermal bonding of the two layers was achieved using a muffle furnace at 590°C for three hours.

The upper layer contained a 3mm diameter central hole for tissue sample placement with three 1.5mm diameter inlets connected via a channel network. The channels measured 190µm in width and 70µm in depth with one as a medium/reagent inlet and two as medium/reagent outlets to aid post-tissue flow (Figure 18). The device was designed using AutoCAD® *(Autodesk Ltd., Farnborough, UK)* computer aided design software at The University of Hull with some adaptations to the procedure described by McCreedy.(388)





Medium was infused into and effluent was collected from the device via 1/16" Tefzel® (Ethylene Tetrafluoroethylene, ETFE) tubing *(IDEX, Wertheim, Germany)* attached to modified trimmed pipette tips (200µl). The inlet tubing was 30cm in length to allow the infused media to be warmed in the incubator prior to perfusing the tissue sample. A flat-bottomed NanoPort[™] hub *(IDEX, Germany)* with an internal diameter of 6.4mm was bonded to the glass over the central hole using epoxy resin (as per the manufacturer's instructions)(Figure 19).



Figure 19) A Microfluidic device with attached NanoPort[™] hub



Figure 19) B Diagrammatic representation of the microfluidic device as adapted from Tanweer *et al.* (389)

A NanoPort[™] reservoir assembly device (*IDEX*) was filled with polydimethylsiloxane (PDMS) (*Dow Corning, Seneffe, Belgium*) and used to create a sealed chamber, allowing gaseous exchange to occur. The PDMS filled adapter was enveloped using a 4cm length of polytetrafluoroethylene (PTFE) (*B&Q, Eastleigh, UK*) tape to ensure an adequate watertight seal within the NanoPort[™] hub.

2.7.2 Priming the device

A syringe connector was attached to the inlet tubing and the device was flushed through the inlet channel with sterile, double distilled, H₂0 to identify any leaks, ensuring that the PDMS filled adapter was removed to allow air within the system to also escape. The adapter was then replaced to flush through the remainder of the device and the outlet tubing. The system was then sterilised by perfusing 70% ethanol *(VWR BDH Prolabo®, Lutterworth UK)* at a rate of 10ml/hour using a calibrated syringe driver *(PHD 2000 Infusion) (Harvard Apparatus, Edenbridge UK)* for 15 minutes. Autoclaved (protocol described in section 2.7.5) double distilled H₂0, which was drawn up within a Class II Biological safety cabinet, was then infused at a rate of 10ml/hour for 15 minutes to remove any residual ethanol.

The microfluidics system was finally perfused with the supplemented DMEM, which was allowed to warm to room temperature before use. A sterile 20ml disposable plastic syringe (*BD™*, *Oxford*, *UK*) was used to draw up the supplemented medium within a Class II Biological safety cabinet. The syringe containing the supplemented DMEM was attached to a Minisart® 0.20µm bacterial filter (*Sartorius, Epsom, UK*) to remove bacterial contamination and minimise bubbles being infused through the microfluidic system, which could potentially cause blockages. This in turn was attached to the inlet tubing via a syringe adapter at a rate of 20µl/minute for at least 30 minutes, to ensure that the medium device was completely filled with the medium (Figure 20). This sterilisation process was repeated with three other devices to ensure that there were a total of four devices for both the control and the radiotherapy-subjected samples. These systems were then transferred to a Class II Biological Safety cabinet to maintain sterile conditions.



Figure 20) Initial infusion of supplemented DMEM via a 0.20µm bacterial filter to remove bacterial contamination and minimise bubbles to prime microfluidic system

2.7.3 Tissue sample preparation

Tissue preparation was also carried out within a Class II Biological Safety cabinet (Esco Airstream[®] E series) *(Esco Global Ltd, Barnsley, UK*) to maintain clean conditions, given the non-sterile nature of colorectal tissue. The collected tissue specimens were either retrieved from the supplemented DMEM if fresh or thawed if previously snap-frozen as described above in section 2.6. The sample was placed in petri dish and cut with a sterile disposable surgical scalpel (*Swann-Morton, Sheffield, UK*) to divide it into single pieces. Each piece was weighed on a laboratory microbalance in grams (to four decimal places), whilst maintaining sterility, and further cut to ensure that it weighed between 0.005g and 0.01g, as previously used by Webster *et al.*(376) Each sample was then placed in individual microfluidic chambers, ensuring that air did not enter the system. A series of parallel microfluidic cultures were routinely established.

2.7.4 Maintenance of tissue sample within microfluidic device

The systems were maintained at 37°±1 °C within calibrated incubators *(Covatutto c/o Hatch-it Incubators, Newbury, UK)*. The sample that would be subjected to external beam radiotherapy was placed inside a constructed Perspex prism (150mm x 120mm x 120mm), which was partially left open using corkboard derived spacer devices to allow airflow over the device and therefore ensure adequate gaseous exchange through the PDMS filled adapter (Figure 21).

The prism was then placed inside the incubator (Figure 22). The supplemented DMEM was infused into the device at a rate of 2μ l/min as per the protocol previously optimised by Hattersley *et al.*(378) The effluent was collected through the outlet tubing two hourly during the day using polypropylene micro-tubes (0.5ml) and also overnight (1.5ml). The collected effluent was stored at 4°C for later analysis.





Figure 21) A & B Perspex glass prism used to deliver external beam radiotherapy. Corkboard spacer used to maintain airflow over microfluidic device. (Microfluidic device within Perspex prism highlighted in green.)



Figure 22) Microfluidic devices maintained within an incubator and perfused with media driven by a syringe driver

2.7.5 Microfluidic system sterilisation

After each experimental run, all reusable components of the microfluidic system (microfluidic device, tubing and syringe connectors) were flushed through with 70% ethanol followed by distilled water to remove any residual media. The components were separated and the modified pipette tips were also removed from the tubing and placed in an empty pipette tip box. This was then placed within an autoclave (Boxer Laboratory Equipment *Ltd, Ware, UK*) for sterilisation. The sterilisation temperature was maintained at 121°C for 30 minutes, followed by a cooling period. The total length of the cycle was two hours for the microfluidic components. (The same protocol was used for liquid sterilisation, however, the complete cycle took a total of two and a half hours due to the extra cooling time). After the cycle was complete, the microfluidic devices were removed immediately and the NanoPorts[™] were lifted off the device. The components were then placed in a drying cupboard for at least four hours. Residual epoxy resin was removed after the individual components were dry and the individual components were reassembled, as described previously in section 2.7.1 prior to commencing further experiments.

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2.8 Optimisation of microfluidic devices with tissue

2.8.1 Initial optimisation with murine colorectal tissue

The intact murine colonic tissue was gently irrigated with the supplemented media to remove any faecal debris and then subsequently divided into smaller pieces (approximately 0.05g). At this point, the tissue was either prepared for interrogation within the microfluidic devices or snap frozen for later use as described in section 2.6. This tissue was used for initial optimisation of procedures.

2.8.2 Maintenance of tissue within a microfluidic device

Parallel microfluidic systems were set up using murine colorectal tissue or human rectal cancer biopsies and effluent collection was carried out. In order to induce cellular rupture and hence infer tissue viability, chemical lysis of the tissue within the microfluidic device was attempted using the Cytotoxicity LDH Kit^{PLUS} kit[™] lysis agent *(Roche[™], Burgess Hill, UK)* or Triton X-100 *(Sigma Aldrich[™], Gillingham, UK)* at a concentration of 10-20% (v/v) in supplemented DMEM. The lysis solution was infused towards the end of the experimental run at a continued rate of 2µl/min through the microfluidic system. Effluent was collected for a further six hours (twohourly collection).

Mechanical lysis was also attempted in addition to chemical lysis to induce a maximal cell death value. This was achieved by removing the PDMS bung and crushing the tissue with non-toothed tissue forceps within the well of the microfluidic device. The bung was replaced and supplemented DMEM containing the lysis agent was perfused through the device. The effluent was then analysed to identify increases in lactate dehydrogenase (LDH) as described in section 2.10.1.

2.9 External Beam Radiotherapy

2.9.1 Radiation Planning

A prism manufactured by the Hull & East Yorkshire hospitals Medical Physics department was designed to house the microfluidics device containing the tissue sample. The dimensions of this device were 120mm x 120mm x 160mm to provide sufficient depth for scatter equilibrium of the incident radiation. Reference marks were made on the surface of the block to act as surrogates of position for set up on the linear accelerator to aid reproducibility.

Computed Tomography scanning of the prism was performed using Philips Brilliance CT Big Bore Oncology configuration[™] (*Philips, Guildford, UK*) to acquire the dimensions of the prism, in addition to aid calculation of the attenuation coefficient in conjunction with CMS XiO® Treatment Planning Software (TPS) (*Elekta, Crawley, UK*). The TPS was then used to plan radiation delivery, in order to apply two beams in a parallel-opposed fashion from the left (90°) and right (270°) lateral sides. A field size of 8cm x 8cm convergent on the isocentre was used, placing the tissue sample at approximately 5.2cm depth from both beams.

An energy dose of 6 Megavolts (MV) was used due to its suitability at such depths. Dose at the centre was normalised to 100% and a prescription of 1Gy was applied. The number of Monitor Units (MU) required was calculated based on the set parameters to delivery the required dose. This was all based on a simple convolution algorithm due to the lack of complex structures inside the prism. Advice on the energy doses and irradiation protocol was provided by Hull & East Yorkshire NHS Hospitals Radiation Physicists, C. Horsfield, K. Hilton and N. Tambe and carried out as previously demonstrated by Carr *et al.*(381)

2.9.2 Radiation delivery

Prior to radiation delivery, the prism was arranged in alignment with the positioning lasers, which coincide with the radiation isocentre. The linear accelerator was then prepared according to the planned parameters and two beams were delivered in succession to provide uniform exposure of the tissue within the microfluidic device. To achieve the requested dose, the MU were multiplied up to the required value.

The incubator containing the microfluidic device encased within the prism, was transferred to the clinical radiotherapy suite (Queen's Oncology centre, Castle Hill Hospital) from the research laboratory, ensuring that the device was continuously perfused throughout the entire period, during both transportation and radiation delivery. The syringe pump and incubator were powered using an uninterruptable power supply, NDG 1500 1500VA Net-Dialog UPS, (*Riello UPS Ltd, Wrexham, UK*) for the duration of the journey (10 minutes +/- 2 minutes) and upon arrival, use of a mains power source was resumed.

Immediately prior to being subjected to radiotherapy, the Perspex prism was removed from the incubator and the prism was fully closed by removing the spacer devices to ensure that the x-rays were accurately delivered to the sample. A linear particle accelerator was used to deliver external beam radiation at dosages between 2Gy and 30Gy. The radiation doses were delivered from two directions by rotating the gantry, so that the total dose was a sum of the two (Figure 23).



Figure 23) External beam radiotherapy delivered using a linear accelerator through the prism within the radiotherapy suite, whilst perfusion of the tissue sample is on-going.

The timing of irradiation was subject to clinical requirements of the radiotherapy suite. Immediately after radiation delivery, the microfluidic device was removed from the prism and returned to the incubator. The system was then returned to the laboratory.

2.9.3 Optimisation of irradiation

Parallel microfluidic systems were set up as described above using murine colorectal tissue or human rectal cancer biopsies. In each of the experimental runs, one of the microfluidic systems was encased within the Perspex prism and irradiated with single fractions of between 2Gy and 30Gy, after allowing acclimatisation of the tissue in the microfluidic device for approximately 24 hours as per Carr *et al.*(381) An initial dose of 2Gy was chosen to approximate the clinical dose of each fraction delivered with neo-adjuvant LCCRT (45Gy over 25 fractions) in the treatment of locally advanced rectal cancer.(118)

2.10 Effluent analysis

2.10.1 Lactate Dehydrogenase (LDH) Analysis

LDH is a stable cytoplasmic enzyme that is present in all cells and is released when plasma membranes are damaged.(390,391) Within the microfluidics systems, LDH was released into the infused supplemented DMEM and collected from the outlet tubing. LDH activity was determined using an enzymatic reaction, where initially LDH catalyses the oxidation of lactate to pyruvate and reduces NAD⁺ to NADH and H⁺. Subsequently, a catalyst solution containing diaphorase transfers the H/ H+ to reduce the tetrazolium salt to formazan. This reaction induces a colour change from pale yellow to red; the level of which is dependent on the quantity of cells with damaged plasma membranes and thus quantity of LDH release (Figure 24). These reactions have been described by Decker *et al* to quantify LDH release from lysed tumour cells.(392) The formazan salt is water-soluble and has a broad absorbance with a maximum at approximately 500nm, whereas the tetrazolium salt has a minimal absorbance at these wavelengths.

Cell death and lysis was quantified by using a colorimetric LDH cytotoxicity assay using a Cytotoxicity LDH ^{PLUS} kit[™](*Roche[™]*, *UK*), following a modified version of the manufacturer's protocol, where half the volume of the reaction mixture was used, as previously performed by Hattersley *et al.*(380,382) An aliquot (50µl) of effluent from each time point, along with a sample of the supplemented DMEM was placed in individual wells of a 96 well cell culture flat bottom microplate (Corning[®] Costar[®]) (*Sigma Aldrich[™]*, *UK*). Providing sufficient effluent was available, this was done in duplicate to calculate a mean value. The lysis kit was prepared to create enough solution for 100 wells by adding 125µl of the reconstituted catalyst solution (lyophilizate, stabilised) to 5.63ml of the dye solution (iodotetrazolium chloride and sodium lactate). Aliquots (50µl) of the resulting reaction mixture were then added to each of the wells.

The 96-well microplate was then placed in the incubator for 30 ± 0.5 minutes. Aliquots (25µl) of the stop solution were then pipetted into each well. The microplate was then read using a Thermo Scientific Multiskan FC microplate spectrophotometer (*Thermo Fisher Scientific, Basingstoke, UK*) at an absorbance of wavelengths 492-620nm as a background correction. The results were analysed on Microsoft Excel 2011 for Mac (*Microsoft, Reading, UK*). These duplicate results were used to calculate a mean value. The mean of the supplemented medium, used as the background control was subtracted from this value. A graph was plotted of time against absorbance per gram of tissue.



Figure 24) Simplified diagram of LDH release assay, where LDH catalyses conversion of lactate to pyruvate and subsequently tetrazolium salt is converted to a formazan salt to induce a colour change that can be measured colourimetrically at 492nm, as adapted from Namiki *et al.*(393)

2.10.2 M30 CytoDeath[™] Enzyme linked immunosorbent assay (ELISA)

During apoptosis, the role of caspases are to cleave various cellular proteins including keratin 18 (K18), a type I cytokeratin that is expressed in single layer epithelial tissue.(394) The M30 neo-epitope exposed after cleavage of K18 is recognised by the M30 antibody.(395) Caspase 3, 7 and 9 cleave K18 to generate the M30 epitope (Figure 25).(396) This cleavage has been shown to occur early in the apoptotic cascade, prior to DNA nick-end labelling. Levels of soluble caspase cleaved K18 fragments containing the M30 neo-epitope can thus be measured using ELISA. In the event of cellular necrosis, there is leakage of full length of K18, rather than its cleaved products.(383)

The immunoassay was used for the quantification of apoptosis associated K18Asp396 (M30) neo-epitope in only the maintained human rectal biopsies. The primary antibody is a mouse monoclonal antibody directed against human, bovine and monkey cleaved cytokeratin and therefore will not detect murine cleaved cytokeratin. Effluent collected during an experimental run prior to and after radiotherapy was analysed for the presence of the M30 neo-epitope. A standard curve was plotted for each ELISA using known concentrations of supplied antigen versus absorbance to subsequently calculate the quantity of antigen in each sample. Absorbance was measured in units/litre (U/l).



Figure 25) Overview of keratin 18 cleavage and M30 antibody activity with M30 antibodies to the exposed M30 neo-epitope after cleavage of K18 during apoptosis, as adapted from Micha *et al.*(380)

Effluent collected at time points prior to and subsequent to irradiation was aliquoted in 60µl volumes into micro-tubes (0.5ml) for each time period. These were immediately transferred to an ultra-low temperature scientific freezer (-80°C). This ensured that a sufficient volume of effluent was available to run duplicates of 25µl.

The M30 CytoDeath[™] ELISA (*PEVIVA AB[™], Sundbyberg, Sweden*) kit was used to perform the immunoassay, as per the manufacturer's instructions. Individual components of the immunoassay were warmed to room temperature (24°±3°C) immediately prior to performing the procedure. The M30 CytoDeath horseradish peroxidase (HRP) conjugate was prepared by adding the M30 CytoDeath conjugate dilution buffer (9.2ml) directly into the vial of M30 Cytodeath HRP conjugate (0.4ml) and mixed thoroughly. The wash tablet contained within the kit was completely dissolved, by adding it to deionised water (500ml).

The M30 CytoDeath standards (range: zero, 0U/l to high 3000U/l) were used to establish further concentrations using the standard zero for dilution. The standards and the diluted effluent (25μ) at the various time points was pipetted into individual wells of the ELISA microplate, ensuring duplication for later calculating a mean. The diluted M30 CytoDeath HRP conjugate (75µl) was immediately added to each well and the microplate was covered with sealing tape. The microplate was then placed on a shaker to allow incubation for four hours to ensure that there was adequate movement of liquid in each well without spillage or at a speed setting of 600rpm. The microplate was subsequently washed five times in a plate washer, using 400µl per well of the prepared wash solution. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (200µl) was added to each well and the microplate was incubated in darkness, at room temperature for 20 minutes ± 1 minute. Stop solution (50µl) was finally added to each well before shaking for five to ten seconds to ensure mixing. After five minutes, the microplate was placed in the microplate spectrophotometer and the absorbance at 450nm was determined as per the manufacturer's instructions.

After initial optimisation, it was noted that some effluent samples had an absorbance value that was greater than the standard high (3000U/l).

Therefore dilution of individual effluent samples with supplemented DMEM was carried out for subsequent immunoassays. Calculation of the original concentration was subsequently obtained by multiplying the measured concentration by the dilution factor.

Finally analytical results were calculated using Microsoft Excel 2011 for Mac *(Microsoft, UK)*.

2.11 Cryosectioning

Individual tissue samples were cryopreserved for sectioning to examine the effects of both maintenance within the microfluidic device and any conditions that they were subjected to, using a technique described by Fischer *et al.*(398) The specimens were prepared on a corkboard mount using Tissue-Tek® O.C.T.™ compound *(Sakura, Thatcham, UK)*, ensuring that all of the tissue was completely encased. Quenching was achieved by immersing the mounted sample in 2-methylbutane *(Sigma-Aldrich™, UK)*, cooled using liquid nitrogen until the optimal cutting temperature (OCT) compound became hard and white in colour for 45 ± 15 seconds. The quenched sample was immediately transferred to a -20°C freezer and stored until sectioning was performed.

Sectioning was performed using a Leica CM1100 Cryostat (*Leica Microsystems, Milton Keynes, UK*). To section the cryopreserved sample, the cork mount was attached to a cryostat chuck using the OCT compound and cooled to -20°C within the cryostat. Sections of 8 to 10µm were cut and placed onto the mounting surface of positively charged slides (StarFrost®) (*Thermo Fisher Scientific, UK*). The slides were labelled with pencil. Sections were left to dry for 20 ± 10 minutes at room temperature before storage within a -20°C freezer.

2.12 Haematoxylin and eosin (H&E) staining

Cellular architecture was visualised following perfusion within the microfluidic device. A standard H&E staining protocol was used as directed

by the Pathology department at Castle Hill Hospital with minor modifications as follows.(399)

Haematoxylin solution (*Sigma-Aldrich™*, *UK*) was poured through filter paper in a funnel into a foil covered bottle prior to use, to remove any precipitates and prevent further oxidation. Methanol 100% (*VWR BDH ProLabo®*, *UK*) was used as a fixative and was cooled at -20°C for at least 30 minutes. The slides were removed from the -20°C freezer and placed in a slide rack to allow sections to warm to room temperature for 5 minutes. All of the slides were labelled with a graphite pencil to avoid dissolution of ink within the methanol. The slide rack was immersed in the ice-cold methanol to fix the sections at room temperature for ten minutes and then immediately rinsed in running tap water for one minute. The slide rack was then placed in filtered Harris Haematoxylin solution (*Sigma-Aldrich™*, *UK*) for one minute before rinsing again in running tap water for a further minute. The slide rack was then transferred into Eosin Y (w/v) 0.5% (*Sigma-Aldrich™*, *UK*) in acidified ethanol 95% for four minutes and followed by a further rinse in running tap water for one minute.

The sections were dehydrated using graded ethanol and subsequently immersed in gradually increasing concentrations of ethanol (70%, 90% and 100%) for a period of two minutes in each. Clearing of the tissue sections was achieved by immersing the slides in three different solutions of Histoclear II[™] (*National Diagnostics[™]*, *Hull*, *UK*) for a period of two minutes in each.

Finally, the slides were mounted using Histomount[™] (*National Diagnostics[™]*, *UK*) and a coverslip (*Thermo Fisher Scientific, UK*) was placed over the sections. After allowing the Histomount[™] to dry, the slides were viewed and digital photography was taken using a Nikon[™] Eclipse E800 (*Nikon UK Ltd, Kingston Upon Thames, UK*) and Image-Pro Premier[™] (*Media Cybernetics, Marlow, UK*).

2.13 Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay

The assay was used to label free 3'-OH terminal DNA strand breaks with fluorescein-deoxyuridine triphosphate (dUTP) modified nucleotides to identify and quantify apoptosis at a single cell level (Figure 26).(400)



Figure 26) Diagram to represent the TUNEL assay principle, as adapted from R&D systems.(401) Nicks in DNA are identified by terminal deoxynucleotidyl transferase (TdT), which catalyses the addition of a deoxyuridine triphosphate nucleotide (dUTP). Secondary labelling with an immunofluorescent marker is then used to label these DNA breaks.

The following solutions were prepared 24 hours prior to commencing the assay:

Tris HCl (tris(hydroxymethyl)aminomethane hydrochloride) 50mM (0.78g) (Trizma[®] HCL) (*Sigma Aldrich[™]*, UK) was dissolved in 100ml of distilled water. After titrating the pH to 7.5 using either sodium hydroxide tablets or HCl 1mM, bovine serum albumin (BSA) (*Sigma Aldrich[™]*, UK) (100mg) was dissolved in the solution.

- Phosphate buffered saline (PBS) was prepared by dissolving one PBS (Dulbecco 'A') (Oxoid[™], Basingstoke, UK) tablet per 100ml of double distilled water as required. The dissolved PBS was autoclaved, as described in section 2.7.5.
- 3) A fixation solution of paraformaldehyde 4% (w/v) was prepared by adding paraformaldehyde (Sigma Aldrich[™], UK) (20g) to the prepared PBS (500ml) within a chemical fume hood and then placed on a hotplate stirrer at low temperature for a minimum of three hours to ensure dissolution. The pH was then titrated to 7.5.
- 4) A permeabilisation solution of 0.1% (v/v) Triton X-100/0.1% (w/v) sodium citrate was prepared by adding Triton X-100 (Sigma Aldrich[™], UK) (0.5ml) and sodium citrate dihydrate (Sigma Aldrich[™], UK) (0.5g) to PBS (500ml).
- 5) Deoxyribonuclease (DNase) I recombinant grade I (Lyophilisate[®]) (*Roche[™]*, UK) (10 000U) was dissolved in 250µl of distilled H₂0 (40 000U/ml) and divided into aliquots (15µl). These individual aliquots were stored at -80°C. Immediately prior to commencing the assay, Tris/HCl solution (200µl) was added to the thawed aliquot of the dissolved DNase I (15µl) to form the DNase I recombinant solution that was used to induce strand breaks.
- 6) The TUNEL reaction mixture was also prepared immediately prior to commencing the assay, ensuring that sufficient volume was available for each slide that required incubation with the solution. This was composed of TUNEL label solution (*Roche™*, UK) (45µl) and TUNEL enzyme solution containing terminal deoxynuclotidyl transferase (TdT) (*Roche™*, UK)(5µl) per slide.

2.13.1 Procedure

The assay technique used was adapted from Gavrieli *et al* and is described as follows.(402) Slides containing the frozen sections were removed from the -20°C freezer and placed in a slide rack. Sections were fixed using paraformaldehyde 4% (w/v) solution, as described above for 20 minutes at room temperature. The slides were washed with PBS for 30 minutes and then incubated in the ice-cold permeabilisation solution for two minutes. Two further five-minute washes with PBS were then carried out and the slides were removed from the rack and laid on a flat surface with the sections facing up.

Sections from tissue that that had not undergone any treatment (i.e. prior to microfluidics) were used as positive controls, to assess that the TUNEL reaction mixture functioned appropriately. These sections were incubated with DNase I recombinant solution 100μ l for ten minutes at room temperature to induce DNA strand breaks prior to labelling. These sections were subsequently washed in PBS for five minutes. Sections from tissue that had undergone treatment (i.e. post microfluidics) were left in PBS while the positive controls were incubated.

The slides were then carefully dried around the sections and the TUNEL reaction mixture (50 μ l) was applied to each of the treatment slides and one of the two positive control slides. To the remaining positive control slide, only the TUNEL label solution (50 μ l) was added and the slide was labelled for later identification with graphite pencil. A coverslip was placed over the sections of each slide (Figure 27).



Figure 27) Diagrammatic summary of TUNEL protocol

The covered slides were placed in an encased humidified chamber at 37°C for one hour in the dark. After removal, the slides were rinsed in PBS twice for five minutes, ensuring that the coverslips were carefully removed. The slides were again carefully dried around the sections and one drop of Vectashield[®] Hard Set[™] mounting medium with DAPI (4',6-diamidino-2-phenylindole) *(Vector laboratories, Peterborough, UK)* was applied and coverslips applied.

The sections were visualised using a Nikon Eclipse E800 fluorescence microscope at an excitation wavelength of 450-500nm and detection wavelength of 515-565nm. Random images were viewed using blue (DAPI) and green (FITC, fluorescein isothiocyanate) filters and digital photographs were acquired using Image-Pro Premier[™]. Image J[™] (National Institutes of Health, Bethesda, USA) was also used to aid quantification of apoptotic ratios.

2.14 Immunohistochemistry

2.14.1 M30 CytoDeath™ immunohistochemistry

Using the same M30 CytoDeath[™] antibody as used in the ELISA, immunohistochemical analysis was also used to quantify levels of caspase cleaved K18 fragments containing the M30 neo-epitope within the human rectal biopsy specimens.

The following solutions were prepared immediately prior to commencing immunohistochemistry (All of the following chemicals were obtained from Sigma Aldrich[™] unless otherwise stated):

- Tris-buffered saline (TBS) was prepared by adding Tris 1M (121g) (Trizma[®]) and sodium chloride (NaCl) 3M (170g) to distilled H₂O to make up to 1L of solution. The pH of the solution was titrated to 7.6 as described in section 2.13.1. This solution was further diluted 20x using distilled H₂O (50mM Tris/150mM NaCl).
- A solution of methanol/hydrogen peroxide (H₂O₂) was formed by adding H₂O₂ 3% (v/v) (40ml) to methanol 100% (360ml). This solution was stored in a bottle with a vented cap and was reused

up to three times for future experiments by adding H_2O_2 (40ml) on each occasion.

3) The primary antibody, M30 CytoDeath[™] (PEVIVA AB[™], Sweden) was diluted at a concentration 1:100 in TBS, ensuring that sufficient volume was available (100µl per slide). The isotype control antibody, MOUSE IgG2b negative control antibody (AbD Serotec, Oxford, UK) was also diluted at a concentration 1:100 in TBS.

2.14.1.1 Procedure

The method used was adapted from previously used techniques and is described as follows.(403,404) Slides containing frozen sections were placed in a slide rack and fixed using 100% methanol that had been cooled at -20°C for at least 30 minutes. Fixation was carried out at -20°C for 20 minutes before washing with TBS for five minutes. Endogenous peroxidases, which are present in multiple cells, including haemoglobin, myoglobin, cytochrome, catalases, along with areas adjacent to vascularised areas, can react with chromagen leading to non-specific background staining.(405) Thus endogenous peroxidase activity was blocked by placing the slide rack into a pot containing the solution of methanol with 3% (v/v) H₂O₂ for 15 minutes and then rinsed in tap water for one minute.

The slides were removed from the slide rack and assembled into a Sequenza[™] rack *(Thermo Fisher Scientific)* after briefly immersing each coverplate in TBS. Each of the reservoirs was filled with TBS and washed for ten minutes. The sections were then incubated for 20 minutes with diluted normal horse serum (Vectastain[™] Elite ABC kit) *(Vector laboratories, UK),* formed from adding one drop (50µl) of the stock solution to TBS (5ml) in the mixing bottle. Using the dropper bottle, a minimum of five drops were added to each slide.

Any non-specific binding of the avidin/biotin system reagents was blocked by adding three drops of the Avidin D solution (Avidin/Biotin Blocking kit[™]) *(Vector laboratories)* to each slide and incubated for 15 minutes. The slides were then rinsed with TBS for ten minutes and subsequently, three drops of the Biotin solution (Avidin/Biotin Blocking kit[™]) (*Vector laboratories*) were added to each slide. After 15 minutes, the slides were again rinsed in TBS for a further ten minutes. The collection of fluid at the bottom of the Sequenza[™] rack was then emptied.

The diluted primary antibody, M30 CytoDeath^M (*PEVIVA AB*^M) (100µl) was added to all slides except for the negative control. One slide containing sections from tissue that had not undergone treatment (i.e. premicrofluidics) was used as a negative control and the isotype control antibody (Mouse IgG2b) (100µl) was added to this slide. After addition of the antibodies, the Sequenza[™] rack was covered and the sections were left at room temperature to incubate for one hour before rinsing with TBS for ten minutes. The diluted secondary antibody (Vectastain[™] Elite ABC kit) (Vector laboratories, UK) was formed from adding two drops (100µl) of the normal blocking serum and two drops (100µl) of the concentrated universal biotinylated anti-mouse/rabbit IgG secondary antibody stock solution to TBS (5ml) in the mixing bottle. The slides were then incubated with the resulting solution for 30 minutes. During this incubation period, the Vectastain[™] Elite ABC reagent was prepared by adding two drops of reagent A (Avidin DH) along with two drops of reagent B (biotinylated HRP H) to TBS (5ml) and mixing immediately. The resulting solution was then allowed to stand for 30 minutes prior to usage. After incubating the sections with the secondary antibody, the slides were rinsed in TBS for ten minutes and then each slide was incubated with 100µl of the prepared Vectastain[™] Elite ABC reagent for 30 minutes. The slides were then again rinsed for ten minutes with TBS.

Amplification of staining and thus improved sensitivity is acquired by increasing the number of enzyme molecules bound to the antigen with the use of the avidin molecule, which has four binding sites (Figure 28).(406) Less of the primary antibody is also required than using direct methods of detection using this method.

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Figure 28) Diagrammatic representation of the avidin/biotin complex (ABC) immunostaining method, as adapted from ThermoFisher Scientific.(406) The primary antibody binds to the target antigen, i.e. M30 CytoDeath™ antibody to caspase cleaved cytokeratin. A biotinylated secondary antibody with specificity against the primary antibody (universal biotinylated anti-mouse/rabbit IgG) is then bound. The avidin-biotinenzyme complex is then added and therefore any vacant biotin-binding sites on the avidin molecule bind to the biotinylated antibody that is bound to the antigen. This results in an increase in signal intensity and sensitivity for detection.

DAB is water soluble in its unoxidised form, but in the presence of peroxidase and hydrogen peroxide, DAB is oxidised, leading to the deposition of an insoluble brown precipitate at the site of enzyme activity. One gold 3, 3'-diaminobenzidine (DAB) tablet and one silver H₂O₂ tablet (Sigma Fast[™] DAB Peroxidase substrate tablet set) (Sigma Aldrich[™], UK) were dissolved in 1ml of distilled H₂O. The slides were removed from the Sequenza[™] rack and laid flat. The area around the sections was dried and a hydrophobic barrier pen (ImmEdge[™]) (Vector laboratories, UK) was used to draw around it. The DAB solution (200 μ l) was then applied to each slide, ensuring that each section was covered, for approximately five minutes or until the colour was sufficiently developed.

The slides were returned to the slide rack and rinsed under running tap water for a period of two minutes and then counterstained using filtered Harris Haematoxylin solution *(Sigma Aldrich™)* for 25 seconds, before again rinsing in running tap water for two minutes.

The sections were dehydrated using graded ethanol. They were immersed in gradually increasing concentrations of ethanol (70%, 90% and 100%) for a period of two minutes in each. The tissue sections were cleared by immersing the slides in three different solutions of Histoclear II[™] for a period of two minutes in each.

Finally, the slides were mounted and analysed as described in section 2.12. After allowing the Histomount[™] to dry, the slides were viewed and digital photography was taken using a Nikon[™] Eclipse E800 and Image-Pro Premier[™] (*Media Cybernetics, UK*). Quantification was performed as per TUNEL analysis described in section 2.13.1.

2.14.2 Cytokeratin

Cytokeratins are intermediate filaments that can be identified within the epithelial cytoplasmic cytoskeleton. Epithelial cell expression of individual subtypes of cytokeratins is dependent on the type of epithelial tissue, as well as the phase of development.

The antibody was used to label epithelial tissues and to identify normal and neoplastic cells of epithelial origin by reacting with cytokeratin 5, 6, 8, 17 and possibly 19.(407) It has been shown to strongly stain epithelial tumours of colorectal origin.(408) The protocol was carried as described in section 2.14.1 as per M30 CytoDeath[™] using the primary antibody, monoclonal mouse anti-human cytokeratin clone MNF116 (*Dako, Ely, UK*), which was diluted at a concentration 1:100 in TBS and its isotype control antibody, MOUSE IgG1 negative control antibody MCA928 (*AbD Serotec, UK*) diluted at a concentration 1:100 in TBS.

2.15 Metabolomic analysis

2.15.1 Sample preparation

Any remaining effluent collected overnight in 1.5ml micro-tubes was frozen to -80°C within an ultra-low temperature scientific freezer. These samples were then transferred to the Centre of Excellence in Mass Spectrometry at the University of York on dry ice for metabolomic analysis. Experimental analysis was carried out by Professor J. Thomas-Oates' team. Although the manner in which this was carried out has not been previously published, the technique was adapted from previous work by the group and based on identification of target metabolite compounds using liquid chromatography – mass spectroscopy (LC-MS).(384,409,410) The aim of this technique was to identify differing compounds between irradiated and non-irradiated samples.

Effluent samples from the same microfluidic device from night 1 (i.e. within 24 hours) were paired with the corresponding effluent samples collected from night 2 (i.e. within 48 hours). These were grouped to ensure that each batch contained paired samples from both control (non-irradiated) microfluidic devices and devices that had been irradiated.

To ensure standardisation of the number of freeze-thaw cycles, each sample was thawed once and divided into 100µL aliquots, re-frozen and stored until extraction at -80 °C. At this point, samples were quickly defrosted, and methanol (300µL) was added to each 100µl aliquot of sample to precipitate soluble protein, followed by immediate vortex mixing for 30 seconds. The resultant solution was incubated at -20°C for 30 minutes and subsequently centrifuged at 13 000*g* for ten minutes at 4°C to pellet the precipitated protein. Double distilled water (150µL) was added to 300µL of supernatant and vortexed for 30 seconds. A quality-control (QC) sample was prepared by mixing aliquots from all samples. These extracts were stored at -80°C until analysis took place.

2.15.2 Mass spectrometry

The low molecular mass metabolite-containing biopsy effluents were analysed using liquid chromatographic separation interfaced directly to mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS) analysis was performed on a Dionex UltiMate[®] 3000 High Performance Liquid Chromatography (HPLC) system *(Thermo Fisher Scientific, UK),* coupled to a maXis[™] HD orthogonal acceleration-time of flight (oaTOF) mass spectrometer *(Bruker, Coventry UK)*.

Either a 10µL aliquot of the prepared sample or QC mixture was injected onto a Dionex Acclaim 120 C18 reverse phase HPLC column (150mm x 2.1µm) (*Thermo Fisher Scientific, UK*). The sample or QC mixture was dissolved in 0.1% (v/v) formic acid in water (*Sigma Aldrich*^m, *UK*) (Mobile phase A), and subsequently dissolved in 0.1% (v/v) formic acid in methanol (*Sigma Aldrich*^m, *UK*) (mobile phase B). From the initial starting conditions of 5% B, the proportion of solvent B was rapidly increased to 16% for three minutes, and increased to 70% over a further three minutes. The proportion of solvent B was gradually raised from 70% to 100% over 12 minutes, and held for 10.5 minutes, before returning to initial conditions for 1.5 minutes to re-equilibrate the system. Mass spectral data was collected in either positive or negative ionisation mode in separate runs, over the mass to charge ratio (*m/z*) range 50-2000.

Samples were randomised and analysed blind, using triplicate injections to control for analytical variability. The HPLC column was conditioned prior to use with ten injections of the QC sample in order to equilibrate the column and increase reproducibility between analyses. High-resolution mass spectrometry was subsequently used to characterise any metabolites identified as being markers of radio-resistance or relevant to understanding the biology of the system.

2.15.3 Multivariate Data Analysis

Raw data files from the Hystar[™] (*Bruker, UK*) data acquisition software suite were converted to the universal mzXML format using the CompassXport

(Bruker, UK) software tool. The mzXML files were then imported to the MZmine 2 software package (available from

http://mzmine.sourceforge.net/index.shtml) for further analysis.(411) An exploratory, rather than a hypothesis-driven approach to data analysis was led by Dr Wilson (Chemometrics expertise, University of York). Unsupervised methods, including principle component analysis were used to identify patterns in the data and potential outliers, and supervised techniques including partial least squares discriminant analysis were used for classification and to determine discriminatory variables. In the case of the supervised learning algorithms, data were reserved for use as an independent test set to validate the results. Variables that provided consistent discrimination between classes (with and without radiotherapy treatment *in vitro*; and clinically radio-resistant *vs.* non-resistant) were analysed further using mass spectrometric approaches to identify the metabolites responsible. Interpretation of the principal component analyses in relation to experimental outcomes was performed by myself with the aid of Professor J. Greenman.

2.16 Summary of assays

A summary of the experimental analyses performed on both the murine colorectal tissue and the human rectal cancer biopsy specimens is demonstrated in Figure 29.



Figure 29) A flowchart to summarise the experimental analyses performed on murine and human tissue.

2.17 Rectal cancer patient demographics and clinical features

Rectal cancer biopsies from eleven patients were evaluated. Histology specimens taken previously, at the time of diagnostic flexible sigmoidoscopy, had confirmed each of these lesions to be an invasive adenocarcinoma. These cancers were staged as T3 or T4 on pre-treatment imaging (by either MRI or CT if contra-indications to undergo MRI) (Table 16).

Seven of the eleven patients in the study underwent neo-adjuvant therapy, with six of these having undergone long course radiotherapy. Concurrent chemotherapy was administered in five of the patients, however, in one patient, chemotherapy was omitted from the regime due to their comorbidities. Of the six patients undergoing long course neo-adjuvant therapy, only five survived to undergo surgical resection. Therefore Mandard TRG was only possible in these five patients. All of these patients had a TRG 3 or 4 with little evidence of histopathological response to neoadjuvant therapy.

Due to the tumour of one patient being deemed resectable on radiological imaging, management consisted of SCRT followed by resectional surgery. One patient was deemed to be of high risk of morbidity and mortality after anaesthetic review and therefore after counselling, underwent a combination of external beam and contact radiotherapy. Biopsies taken to assess response did not identify any evidence of malignancy and the patient is continuing clinical follow up.

Two patients were treated palliatively in view of the findings of advanced disease at attempted curative surgery. Both patients were managed with defunctioning stomas followed by palliative chemotherapy or chemoradiotherapy. The remaining patient chose not to undergo neo-adjuvant therapy and went straight to resectional surgery. The clinical outcome data is summarised in table 17.
Patient	Age at	Sex	Pre-	Pre-	Pre-treatment
	diagnosis		treatment	treatment	histology
			staging based	cytology	
			on MRI/CT		
1	65	Male	T4, N2, M1	Not	Invasive
				performed	adenocarcinoma
2	67	Male	T2/3, N1, M0	C5	Invasive
					adenocarcinoma
3	55	Male	T3a, N2 M1	C5	Invasive
					adenocarcinoma
4	78	Male	T4, N2, M0	C5	Invasive
					adenocarcinoma
5	81	Male	T3a, N2, M0	C4	Invasive
					adenocarcinoma
6	77	Female	T3c, N1, M0	C5	Invasive
					adenocarcinoma
7	75	Female	T2/3, N0, M0	C5	Invasive
					adenocarcinoma
8	59	Male	T3, N1, M0	C5	Invasive
					adenocarcinoma
9	53	Male	T3/4, N1, M0	C4	Invasive
					adenocarcinoma
10	63	Male	T3, N1, M0	C5	Invasive
					adenocarcinoma
11	63	Female	T3, N0, M0	C4	Invasive
					adenocarcinoma

Table 16) A summary table of demographics and pre-treatment staging ofpatients from whom rectal biopsies were taken for microfluidic analysis.

(T – tumour, N – nodal, M – metastases; Cytology C1 – C5 with C4 - suspicious of malignancy & C5 - malignant)

Chemo/radiotherapy Surgical procedure Histology at Patient Mandard Fraction(s) - # resection TRG Palliative chemotherapy (oxaliplatin & capecitabine) Defunctioning (loop) colostomy (due to locally N/A N/A 1 advanced rectal cancer & disseminated peritoneal disease) 2 SCRT 25Gy/5# Anterior resection + limited right vpT2, N0, Mx N/A hemicolectomy for synchronous (low grade) (Dukes B1) tubular adenoma unsuitable for endoscopic removal Palliative radiotherapy 25Gy/5#/palliative chemotherapy Defunctioning ileostomy + tube caecostomy N/A N/A 3 (12 cycles oxaliplatin + modified de Gramont chemotherapy + 6 cycles irinotecan) ypT4, N1, Mx LCCRT (45Gv/25# + capecitabine)TRG 4 4 Pelvic exenteration (Dukes C2) LCCRT (45Gy/25# + capecitabine) 5 APER ypT3, N2, Mx TRG4 Adjuvant chemotherapy (Dukes C2) Long course radiotherapy (45Gy/25#)/chemotherapy APER ypT3, N1 Mx TRG4 6 omitted due to co-morbidities (Dukes C2) 7 Contact radiotherapy to rectum 90Gy/3# Nil N/A N/A External beam radiotherapy 25Gy/5# LCCRT (45Gy/25# + capecitabine)N/A N/A 8 Died prior to surgery 9 LCCRT 45Gy/25# + capecitabine) Pelvic exenteration ypT4, N2, Mx TRG 3/4 (Dukes C2) LCCRT (45Gy/25# + capecitabine)TRG4 10 vpT3, N2, Mx Low anterior resection (Dukes C2) Nil N/A 11 vpT3, N0, Mx Anterior resection (Dukes B2)

Table 17) A summary table of patient treatment, subsequent clinical response and tumour response according to Mandard TRG

(LCCRT – long course chemoradiotherapy; TRG – tumour regression grade; # - fractions)

2.18 Statistical analysis

Statistical analysis was performed using both Microsoft Excel for Mac 2011 (*Microsoft, UK*) and GraphPad QuickCalcs (*GraphPad Software, Lo Jolla, USA*). Paired and unpaired t-tests were used to determine if marker release was significantly altered. A p value of ≤ 0.05 was considered to be significant and thus reject the null hypothesis.

SECTION 3: Results

3.1 Patient recruitment

As stated in section 2.2.1, although it was anticipated that at least fifty patients would undergo LCCRT based on hospital throughput over the two-year study period (2012-2014), not all of these patients presented through the two week wait clinics. Although the possibility of further recruitment at other hospitals was discussed, this was decided against to ensure that the stringent protocols in tissue preparation were maintained. Hence an amendment was submitted to the REC to also enable collection of rectal cancer tissue at the time of surgery in patients that had not undergone neo-adjuvant therapy, to increase the number of patients available to the study (section 2.2). In total, twenty-three patients were recruited, however only the samples of twenty-one patients were used for analysis. Although a total of twenty-nine patients were eligible, two patients refused participation and a further four agreed to participate, but their biopsies were unable to be collected, due to their colonoscopies being carried out at another hospital site, which would alter the stringent collection protocol used.

3.2 Optimisation of microfluidic devices with tissue

3.2.1 Optimisation with murine colorectal tissue

Parallel microfluidic systems were initially set up using murine colorectal tissue (n = 8 devices) and effluent collection was carried out for 70 hours as previously described in section 2.8. Overnight (range 10-16 hours) effluent collections were not analysed for LDH release, as they were deemed not to be comparable with the other two hourly collections. Instead this effluent was used for metabolomics analysis as described in section 2.15. Using the raw data obtained from Multiskan FC microplate spectrophotometer, a mean was calculated based on the values obtained from duplicate samples. The absorbance value (*A*) of the supplemented media being perfused through the system was then subtracted from this mean and finally the absorbance per gram was calculated based on the wet weight of the biopsy (in grams) interrogated.

LDH level (A**/g)** = [(A effluent^a + A effluent^b)/2 – A media]/biopsy weight A worked example of the calculation used to plot graphs is shown in table 18.

Table 18) Table demonstrating calculation of LDH release per gram oftissue based on absorbance at 492nm of two microfluidic devicesmaintaining murine colorectal tissue.

	Mean A at	492nm	(Mean A at 492nm - A media) per					
	(based on two		gram of tissue					
	duplicates)						
Incubation			Tissue					
time (hours)			Α	Tissue B				
	Tissue A	Tissue B	(0.01g)	(0.0088g)	Mean	SD		
0-2	3.48	3.38	249.9	272.0	261.0	15.7		
2-4	3.86	3.59	288.1	295.9	292.0	5.52		
4-6	1.50	2.60	51.3	183.6	117.5	93.6		
20-22	0.900	0.962	0	0	0	1.69		
22-24	1.16	1.45	17.3	52.5	34.9	24.9		
24-26	2.11	1.31	112.3	37.0	74.7	53.2		
26-28	1.76	1.73	77.7	84.9	81.3	5.08		
44-46	0.867	0.960	0	0	0	1.85		
46-48	0.868	0.991	0	0.909	0.455	0.643		
48-50	1.05	1.04	6.5	6.48	6.49	0.0161		
68-70	0.779	1.43	0	50.3	25.2	35.6		

The absorbance of the media in this example is 0.985. Note that any values calculated to be negative were considered to be zero. (Numbers to 3

significant figures or 1 decimal point) (A is the absorbance value)

Measured LDH levels of the effluent were always high within the first few hours of tissue being placed within the microfluidic device and fell to almost negligible levels (<50units/g of wet weight tissue) within a median time of 28 hours (range 22-44 hours) (n = 16 devices). Using paired p-tests to assess alterations in LDH release between the two time points, demonstrated this decrease to be reproducibly significantly (p < 0.05). Lysis of the tissue within two of the microfluidic devices was then attempted in order to demonstrate cell death and hence imply tissue viability. Attempted lysis was initially performed by perfusing the tissue with a solution of 10% v/v lysis agent (Cytotoxicity LDH Kit^{PLUS} kitTM)(*Roche*) in supplemented DMEM. However, notable increases in LDH level were not demonstrated (Figure 30). It is worth noting that due to the low number of replicate samples that there is a possibility of statistical error, particularly type II errors, where the null hypothesis is actually false, but is incorrectly accepted.



Figure 30) Representative graph of LDH release with time one of the two experimental runs, where murine colorectal tissue was perfused for greater than 70 hours with attempted lysis of tissue at 70 hours with a solution of 10% v/v lysis agent (Cytotoxicity LDH Kit^{PLUS} kitTM). Measurement of LDH release was correlated with time. Initial levels of LDH release were demonstrated to be high before falling significantly to negligible levels (<50 units/g of wet weight of tissue) by 24 hours (p = 0.02; paired t-test). No notable increase in LDH seen after attempted lysis at 70 hours (p = 0.75). (control tissue n = 3 devices & lysed tissue n = 1 device in each experimental run) (Standard Deviation shown in error bars)

Murine tissue that had undergone maintenance within a microfluidics device for the same time period, where lysis had not been attempted was cryopreserved and stained with H&E. Cellular architecture was still maintained in this control tissue with evidence of colonic crypt preservation. The tissue did not demonstrate evidence of central necrosis, confirming the diffusion of nutrients and waste products (Figure 31). This suggested that this tissue was still viable with the lysis agent not inducing effective cell rupture.



Figure 31) Representative images of tissue of:

- a) Murine colorectal tissue stained with H&E that was not placed in a microfluidics device (i.e. time after 0 hours) (n = 8 devices)
 - i. x 100 magnification
 - ii. x 400 magnification
- b) Murine colorectal tissue stained with H&E after maintenance in a microfluidics device after 52 hours where lysis was not attempted (n = 6 devices)
 - i. x 100 magnification
 - ii. x 400 magnification

3.2.2 Optimisation with human rectal tissue

These experiments were replicated with human rectal cancer biopsies (n = 20 devices) from ten patients and effluent collection was carried out for up to 98 hours (range 53-98 hours). Measured LDH levels of the effluent were again high within the first few hours of tissue being placed within the

microfluidic device and fell significantly to almost negligible levels (<50units/g of wet weight of tissue) within a median time of 24.5 hours (range 6-70 hours) (p < 0.05). Lysis of the tissue (n = 10 devices) was then attempted in the same manner using the 10% (v/v) lysis agent solution at a median time of 72.5 hours (range 62-94 hours). However, in a similar manner to murine tissue, minimal or no increase in LDH levels were demonstrated within six hours of attempted lysis (Figure 32). Experiments were repeated with the human rectal cancer tissue (n = 2 devices) using a solution of increased concentration, 20% (v/v) lysis agent, but despite this, notable increases in LDH were not observed.



Figure 32) Representative graph of_LDH release with time of one of the experimental runs, where human rectal cancer biopsies (n = 3 devices) were perfused for greater than 70 hours with attempted lysis of tissue at 70 hours with a solution of 10% v/v lysis agent (Cytotoxicity LDH Kit^{PLUS} kitTM). Measurement of LDH release was correlated with time. Again, initial levels of LDH release were demonstrated to be high, before falling significantly to negligible levels (<50 units/g of wet weight of tissue) by 24 hours (p < 0.01; paired t-test). No notable increase in LDH was seen after within six hours of attempted lysis at 70 hours (p = 0.27). (Standard Deviation shown in error bars)

In view of the failure to effectively induce LDH release, the experiments were repeated (n = 4 devices) using a solution of 10% v/v Triton X-100 as a lysis agent. However, again notable increases in LDH were not observed. After attempted lysis, the tissue was snap frozen, sectioned and later stained with H&E. Although LDH levels had not demonstrably increased, losses in cell architecture were seen (Figure 33).



Figure 33) Representative images of human rectal cancer tissue stained with H&E of:

a) After maintenance in a microfluidics device for 66 hours (x 400 magnification) (n = 10 devices);
b) After maintenance in a microfluidics device for 72 hours after undergoing lysis with a solution of Triton X 10% at 66 hours (x 400 magnification) (n = 4 devices)

A combination of mechanical and chemical lysis was tested to induce cellular rupture in view of the minimal alterations in LDH release after using the above lysis agents exclusively. Parallel microfluidic systems (n = 12 devices) were set up above using murine colorectal tissue (n = 4 devices) or human rectal cancer biopsies (n = 8 devices) and mechanically and chemically lysed simultaneously. Mechanical disruption was achieved by crushing the tissue within, as described in section 2.8.2, whilst a solution of 10% (v/v) Triton X-100 was also perfused simultaneously, at a median time of 72 hours (range 72-90 hours) after initiating the experiment. Increased LDH release was reproducibly observed within two hours in both murine and human tissue samples undergoing this form of lysis (Figure 34). Due the nature of mechanical lysis, there was minimal remnant tissue within the device suitable for cryopreservation and therefore assessment of tissue architecture was not possible.

Paired t-tests were used to assess differences in LDH release between the control and the irradiated tissue at two hours after irradiation. Although the increase in LDH release after lysis was found to be significant in the murine colorectal tissue (p = 0.05), significance was not reached with the human rectal cancer biopsies in either of the two experimental runs (p = 0.15 & p = 0.09), which may be attributable to the low number of replicate samples used and the possibility of type II errors.

Initial optimisation experiments where LDH release did not notably increase with attempted lysis could be suggestive of preceding tissue death prior to lysis and thus why a significant rise in LDH was not elicited. However, from the increase in LDH release after simultaneous mechanical and chemical lysis, it can be inferred that a substantial proportion of both murine and colorectal tissue is still viable after maintenance in a microfluidic device at 72 hours. This work has also demonstrated that the tissue was not effectively lysed with chemical lysis as a sole modality.



Figure 34) Representative graphs of LDH release with time of single experimental runs where tissue was perfused for greater than 70 hours with simultaneous mechanical and chemical lysis (with a solution of 10% (v/v) Triton X-100) at 72 hours. Increased in levels of LDH release noted after tissue manipulation within two hours of lysis. (Standard Deviation shown in error bars)

a) Murine colorectal tissue (control tissue n = 3 devices & lysed tissue n = 1 device)_(p = 0.05);
b) Human rectal cancer tissue (control tissue n = 2 devices & lysed tissue n = 2 devices) (p = 0.15)

3.3 Microfluidic device blockages

Although repeated attempts (n = 10 devices) were made to maintain the tissue for longer periods of time, this was limited by blockages of the microfluidic chip. Upon examination of the tissue within the well, it was reproducibly noted that there had been some macroscopic disintegration of the tissue sample with loss of tissue turgidity, thus leading to potential obstruction of inlet and/or outlet channels. Despite this, there were no large increases in LDH release (data not shown).

In the event of a blockage occurring prior to the planned completion of the experimental run, the device was abandoned without an attempt to salvage the specimen by either unblocking the chip or transferring the tissue, in order to preserve the stringent protocols in place and prevent potential false alterations in markers. In view of this, each experimental run contained a minimum of four microfluidic devices to ensure spare devices in the event that one of these may have to be abandoned.

3.4 Radiation delivery optimisation

Murine colorectal tissue was used to optimise radiation dose planning for use on patient samples. Single doses of between 2Gy and 30Gy were delivered to the tissue within the microfluidic devices as described in section 2.9. These specimens were maintained for a total median time of 52 hours (range 49-53.25 hours). The tissue (n = 7 devices) was exposed to a single dose of external beam radiation at a median time of 28.75 hours (range 23-29.25 hours) and then perfused for a further median time of 24 hours (range 23-29 hours) to establish response. This timing was based on the availability of the clinical radiotherapy suite, so as not to impact on scheduled patient appointments. Control non-irradiated tissue (n = 24 devices) was also maintained for the same period of time in order to draw comparisons.

An irradiation delivery time of approximately 24 hours after initial set up was chosen based on the optimisation work shown in section 3.2, which

demonstrated initially high levels of LDH release on placement of tissue into the device and thus allow this to settle. In addition, it is likely that there is continual cell death throughout maintenance of the tissue within the microfluidic device and therefore to ensure maximal survival, the aim was to reduce the overall time of the experimental run.

3.4.1 Optimisation of LDH response to irradiation with murine tissue

Irradiated murine colorectal tissue exposed to doses of radiation of either 2Gy (n = 2 devices) or 10Gy (n = 2 devices) across four experimental runs did not induce LDH release within a few hours of irradiation as previously demonstrated following combined chemical and mechanical lysis (p = 0.5 and p = 0.22 respectively). This is demonstrated in Figure 35, but due to the variable times of effluent collection and radiation delivery, this data cannot be combined and displayed on the same graph.

Given this lack of LDH release in response to lower doses of radiation, a dose of 30Gy was tested. This increased dose reproducibly led to a transient, but marked increase in LDH release in the murine colorectal tissue immediately (within two hours) after exposure to external beam radiation (n = 3 devices) (Figure 36). However only significant increases were only achieved (p < 0.05) in two of the three devices irradiated, with the increase in LDH in the remaining tissue not achieving significance (p = 0.82). As discussed above, this may be due to the possibility of type II errors with this low number of replicate samples.

As demonstrated using various lysis agents in section 3.2, despite minimal or no increases in LDH release, irradiated murine colorectal tissue did however, reproducibly lose architectural integrity (n = 6 devices), when stained with H&E at all radiation doses (2Gy, 10Gy and 30Gy). Colonic crypts were preserved after maintenance within the microfluidic devices, however, radiation exposure led to loss of these well-defined structures. This was not noticeably different between increasing levels of radiation exposure (Figure 37).



Figure 35) Representative graphs of LDH release with time of two experimental runs where murine colorectal tissue was perfused for greater than 24 hours prior to irradiation and subsequently maintained for approximately 24 hours (control tissue n = 3 devices & irradiated tissue n = 1 device in each run). No demonstrable increase in LDH levels was observed after exposure to a dose of **a**) 2Gy radiation at 28.5 hours (p = 0.5); **b**) 10Gy at 29 hours respectively (p = 0.22). (Standard Deviation shown in error bars)



Figure 36) Representative graphs of LDH release with time of two experimental runs where murine colorectal tissue was perfused for greater than 24 hours prior to irradiation and subsequently maintained for approximately 24 hours_ (control tissue n = 3 devices & irradiated tissue n = 1 device in each run). An increase in LDH levels was demonstrated within two hours of exposure to a dose of 30Gy radiation at **a)** 29 hours (*p* = 0.033); **b)** 29.25 hours (*p* = 0.033) respectively. (Standard Deviation shown in error bars)



Figure 37) Representative images of murine colorectal tissue after maintenance within a microfluidic device demonstrating morphological changes when stained with H&E after irradiation (left panel x100 magnification; right panel x 400 magnification):

a) Prior to maintenance, b) Non-irradiated tissue observed after 52 hours, c) Irradiated tissue (2Gy) observed after 52 hours (n = 2 devices), d) Irradiated tissue (10Gy) observed after 52 hours (n = 2 devices) and e) Irradiated tissue (30Gy) observed after 53 hours (n = 3 devices). Although LDH levels were not markedly increased upon irradiation with 2Gy and 10Gy doses, tissue damage with loss of well-defined crypt structures was evident on H&E staining. High levels of LDH release were only induced by very high levels of radiation (30Gy).

3.5 Irradiation of rectal cancer biopsies

Based on the morphological changes induced by a 2Gy dose of radiation in murine colorectal tissue and its approximation to the single fractionated doses delivered in LCCRT, it was decided that subsequent human rectal cancer samples would be interrogated with single 2Gy doses of radiation.(118) Therefore, other potential markers with greater sensitivity of response to irradiation were sought.

Pre-treatment rectal cancer biopsies obtained at colonoscopy from eleven patients identified with rectal cancer were used for analysis. These biopsies were maintained for a median time of 50 hours (range 48-53 hours). The biopsies (n = 11 devices) were exposed to a single 2Gy dose of external beam radiation at a median time of 27 hours (range 23.5-30 hours) and then perfused for a further period of time (median 24 hours; range 22-24.5 hours) to establish response. Control, non-irradiated biopsies (n = 33 devices) were also maintained for the same period of time for comparative purposes.

3.6 Methods of analysis of patient samples

It was decided that the results would be analysed using two approaches:

- For each patient sample, the non-irradiated, control devices were directly compared with the irradiated devices in attempt to identify patients that may have radiosensitive rectal tumours.
- Non-irradiated, control biopsies were grouped and compared with irradiated tumour biopsies to identify the effect of irradiation on rectal cancer biopsies.

Paired t-tests were used to perform a comparative analysis between the

non-irradiated and irradiated devices for each patient and unpaired t-tests were used for comparison of grouped samples. A minimum of three values were used to perform this analysis.

3.7 LDH response of irradiated rectal cancer biopsies

Patterns of LDH response to human rectal cancer tissue were seen to be variable with three groups of patterns seen. Paired t-tests were used to assess differences in LDH release between the control and the irradiated tissue at various time points after irradiation. In the first group, four of the eleven patient samples interrogated (patients 3, 6, 8 & 9) elicited minimal increases in LDH release, which occurred within four hours of being irradiated (Figure 38a). These were not found to be significant. The second group contained three of the patient samples (patients 2, 4 & 10), which demonstrated minimal LDH increases, but these increases were not observed within those initial four hours after irradiation and were noted during the following day (Figure 38b). This rise was found to be significant in only one of these patients, but as activity prior to this point was not assessed, it is difficult to determine the importance of this finding. Finally, in the third group, the remaining four samples (patients 1, 5, 7 & 11) did not demonstrate increases in LDH after irradiation, which may be suggestive of radioresistance (Figure 38c).

Due to a combination of variability in response to irradiation and low numbers of patients in each group, comparison of grouped patient samples was not performed.

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Figure 38) Representative graphs of LDH release of single experimental runs where human rectal cancer tissue was perfused for greater than 24 hours prior to irradiation and subsequently maintained for approximately 24 hours (control tissue n = 3 devices & irradiated tissue n = 1 device in each run). Three patterns of LDH release after irradiation were demonstrated in the eleven patient samples interrogated:

a) Group 1: Representative graph of four patients where minimal increases in LDH levels were observed four hours after exposure to a dose of 2Gy radiation (p = 0.099). Irradiation of tissue at 25 hours. (control tissue n = 3 devices & irradiated tissue n = 1 device)

b) Group 2: Representative graph of three patients where minimal increases in LDH levels were observed 24 hours after exposure to a dose of 2Gy radiation (p = 0.085). Irradiation of tissue at 27 hours. (control tissue n = 3 devices & irradiated tissue n = 1 device)

c) Group 3: Representative graph of four patients where an alteration in LDH levels 24 hours was not observed after exposure to dose of 2Gy (p = 0.5). Irradiation of tissue at 29 hours (control tissue n = 3 devices & irradiated tissue n = 1 device)

(Standard Deviation shown in error bars)

Despite this variability in LDH release between different patient rectal cancer samples when exposed to radiation, architectural losses were identified in all irradiated samples (n = 11 devices) upon H&E staining. Although a degree of cell loss was observed after maintenance within the microfluidic devices, colonic crypts were preserved, as seen previously in murine tissue after maintenance within the microfluidic devices. However, radiation exposure consistently led to loss of these well-defined structures (Figure 39).

Although delivery of single doses of 2Gy radiation were shown to reproducibly destroy tissue architecture after maintenance in the microfluidic devices for a median time of 50 hours (range 48-53 hours), only negligible increases were seen in LDH release in seven of the eleven tissue samples.

Given this variability in the peak of LDH release after irradiation, in addition to quantifying what actually constituted a relevant increase, LDH was deemed to be unsuitable as a sensitive marker of response to irradiation. Therefore, other potentially more sensitive markers were sought to attempt to quantify response.



Figure 39) Representative H&E stained images of rectal cancer tissue from four of the patients (Rows a, b, c, d) to demonstrate morphological changes after irradiation with 2Gy (x400 magnification)

- i) Prior to microfluidics
- ii) Non-irradiated tissue observed after a median time of 50 hours
- iii) Irradiated tissue (2Gy) observed after median time of 50 hours

3.8 M30 Cytodeath[™] ELISA

As this is a preliminary study, only a limited number of time points across the timeframe from each sample were chosen for analysis, with only one control and one irradiated sample analysed for each patient. In our initial five patient samples, we chose to focus on the central time points around the period of irradiation, however, in the six patient samples where early time points (<10 hours) were evaluated, initial released levels of caspase cleaved cytokeratin were demonstrated to be high within the first few hours of the tissue sample being placed within a microfluidic device. (Figure 41a) This pattern of release bears resemblance to the activity of LDH release demonstrated in section 3.2.

A standard curve was plotted for each ELISA using the calibration reagents in the M30 Cytodeath^M ELISA kit. Using a line of best fit, the coefficient of determination (R²) was calculated along with the regression analysis equation (y = ax + b), where y is the calculated concentration (y-intercept), a is the regression co-efficient, x is the mean measured absorbance at 450nm and b is the regression constant. An example is shown in Figure 40. Using the raw data obtained from Multiskan FC microplate spectrophotometer, a mean was calculated based on the values obtained from duplicate samples. The absorbance value (A) of the supplemented media being perfused through the system was then subtracted from this mean. The regression equation was used to calculate concentrations, measured as units per litre, of caspase cleaved cytokeratin based on absorbance values according to wet weight of the interrogated tissue.

Mean concentration of caspase cleaved cytokeratin (Units/l/mg)

= $[a[(A \text{ effluent}^a + A \text{ effluent}^b)/2 - A \text{ media}] + b]/biopsy weight}$



Figure 40) An example of the standard curve plotted for each M30 ELISA that was used to calculate the regression analysis equation. A line of best fit was used to calculate the coefficient of determination (R^2), in addition to the regression analysis equation (y = ax + b), where y is the calculated concentration (y-intercept), a is the regression co-efficient, x is the mean measured absorbance at 450nm and b is the regression constant.

Overall two patterns of activity were observed in assessing the release of caspase cleaved cytokeratin after irradiation. In the first group, seven of the eleven human tissue samples analysed demonstrated no increase in apoptosis after irradiation (Figure 41a). The second group contained the remaining four patient samples, which demonstrated increased levels of apoptosis immediately (within four hours of irradiation) (Figure 41b).



Figure 41) Representative graphs of caspase cleaved cytokeratin release of two experimental runs demonstrating variable patterns in human rectal cancer samples after irradiation:

a) Group 1: No increase in apoptosis observed within 8 hours of exposure to a dose of 2Gy radiation at 23.5 hours. (control tissue n = 1 device & irradiated tissue n = 1 device; representative of seven patients)
b) Increase in LDH levels 4 hours after exposure to a dose of 2Gy radiation at 25 hours. (control tissue n = 1 device & irradiated tissue n = 1 device; data representative of four patients)
(Standard Deviation shown in error bars)

In order to make quantitative comparative assessments between the nonirradiated, control and irradiated samples, the value of caspase cleaved cytokeratin was calculated using effluent collected immediately prior to irradiation and then using effluent collected after irradiation of the device. Paired t-tests were performed to assess if the increases in levels of caspase cleaved cytokeratin after irradiation were significant (p < 0.05) by assessing release in both the non-irradiated and irradiated samples at the first assessed time point (two to fours) after irradiation.

In four of the patients assessed, levels of apoptosis were noted to significantly increase after irradiation (figure 42). A likely anomalous result was seen in the control sample of patient 10, where caspase cleaved cytokeratin release was seen to be considerably higher (>2500U/l/mg) than other irradiated and non-irradiated samples at these time points and also out of scale for the highest standard, thus analysis was not truly accurate (data not displayed). Therefore statistical analysis was not performed on this sample.



Figure 42) Bar chart summarising caspase cleaved cytokeratin release after irradiation with 2Gy amongst the eleven patient samples interrogated. (Note that 'pre' concentrations from both the control and irradiated tissue were analysed using effluent collected in the preceding two hours prior to the irradiated sample receiving a single 2Gy dose of radiation. 'Post' concentrations were analysed using the effluent collected at two or four hours after the sample was irradiated.)

Data not displayed for patient 10, due to a likely anomalous result seen in the control sample where caspase cleaved cytokeratin release was seen to be considerably higher (>2500U/l/mg) than other irradiated and non-irradiated samples at these time points.

Using paired t-tests, significant increases in apoptotic ratio of the irradiated tissue compared to control tissue are shown in bold. (Standard Error of the Mean shown in error bars)

Grouping of patient samples was subsequently performed to compare nonirradiated, control samples with irradiated samples. The patient samples were grouped into low, medium and high levels of apoptosis according to the concentration of the samples at the time point preceding the irradiated sample receiving radiation. (Note the highest value for each patient paired concentration between control and irradiated samples was chosen for grouping purposes.) Concentration groups were classified as follows: Low <100 Units/l/mg (n = 4 patients), Medium 100-500 Units/l/mg (n = 4 patients) and High >500Units/l/mg (n = 3 patients). An example of group allocation is demonstrated in table 19. (Raw data shown in full in appendix 7).

Table 19) An example to demonstrate group allocation in each of the three groups: low (<100 Units/l/mg), medium (100-500 Units/l/mg) and high (>500Units/l/mg) levels of apoptosis according to the concentration of the samples at the time point preceding the irradiated sample receiving radiation.

	Control tissue concentration			Irradiated tissue concentration			
	(Units/I/mg)			(Units/I/mg)			
Patient			Change in	Pre	Post	Change in	
sample	Pre	Post	concentration	irradiation	irradiation	concentration	
Low A	51.1	48.7	-2.45	21.5	197	176	
Low B	<u>51.7</u>	47.5	-4.28	24.7	215	191	
Medium							
А	28.3	27.6	-0.700	191.3	110.0	-81.8	
Medium							
В	24.8	26.9	2.10	<u>213.7</u>	137.0	-76.8	
High A	74.1	37.9	-36.3	492.4	1053.4	561.0	
High B	79.5	26.6	-52.9	<u>511.9</u>	1173.5	661.6	

Note the highest value for each patient paired concentration between control and irradiated samples was chosen for grouping purposes and is underlined.

Although a mean increase in caspase cleaved cytokeratin was observed in the irradiated tissue samples when compared to the control samples in the low release group, this was not found to be significant using an unpaired t-test (p = 0.0613) (Figure 43). No statistical difference was identified between the two groups in the medium apoptosis group (p = 0.258) or the high apoptosis group (p = 0.390), even after exclusion of the anomalous result identified in patient 10 (p = 0.693).



Figure 43) Bar chart summarising alterations in caspase cleaved cytokeratin release after irradiation with 2Gy amongst the three grouped patient samples interrogated (allocation of groups described in section 3.8, table 19. The patient samples were grouped into low, medium and high levels of apoptosis according to the concentration of the patient samples prior to the irradiated sample receiving radiation. (Standard Error of the Mean shown in error bars)

Analysis of these patient tumours individually; where there was an increase in caspase cleaved cytokeratin release after irradiation may be a potential indicator of response to radiotherapy *in vivo*.

In the grouped analysis, when focusing on the low concentration group, there was a trend towards increased apoptosis in the irradiated group when compared to the control group, however, this did not reach levels of significance. This trend was not seen in the medium or high concentration groupings.

3.9 TUNEL assay

Analysis using TUNEL immunohistochemical assay has been previously conducted by several authors by calculating individual apoptotic cells.(412– 414) Due to the coalescence seen between individual apoptotic cells, this was deemed to be an unsuitable method, as it was likely that cells would be undercounted, therefore, it was decided that calculation of the percentage area of each image captured at x400 magnification identified to be undergoing apoptosis would be preferable. Using this modified method of quantification throughout ensured standardisation.

3.9.1 Optimisation of the TUNEL assay in murine colorectal tissue

The murine colorectal tissue irradiated with single doses of 2Gy (n = 2 devices), 10Gy (n = 2 devices) or 30Gy (n = 3 devices) was used to initially assess if levels of apoptosis were increased after irradiation. (Figure 44) This demonstrated a trend towards increased levels of apoptosis on microscopic examination in the murine tissue irradiated at all doses (2, 10 & 30Gy) when compared to the non-irradiated control.

a) Non-irradiated control tissue



Figure 44) Representative images of murine tissue (n = 2 devices) stained using the TUNEL assay after maintenance within a microfluidic device for 52 hours (x400 magnification). A trend towards increased apoptosis was demonstrated after irradiation with 2Gy.

- a) Non-irradiated tissue
- b) Irradiated tissue exposed to a 2Gy dose after 29 hours of maintenance within the microfluidics chamber
 - i) Blue nuclei DAPI counterstained
 - ii) Green nuclei FITC labelled apoptotic nuclei
 - iii) Composite image

Quantification was performed by initially transforming the DAPI counterstained and the FITC labelled apoptotic nuclei images into black and white using Image J[™] (Figure 37).(415) A minimum of three randomly selected areas from each biopsy were used for quantification. Counting was performed using Image-Pro Premier[™] and subsequently apoptotic ratios were calculated.



Figure 45) Representative images of murine tissue irradiated with a single 2Gy dose stained using the TUNEL assay after maintenance within a microfluidic device for 52 hours. These images were_transformed into a black and white image for quantification (x400 magnification):

- a) DAPI counterstained
- b) FITC labelled apoptotic nuclei

Paired t-testing of each control and irradiated sample was performed using the randomly selected areas.

Apoptotic ratio = (Area of FITC labelled apoptotic nuclei/Area of DAPI counterstained nuclei) x 100
An example of the data used to perform this calculation is shown in table 20. **Table 20)** A table to demonstrate how raw data was used to assess significance using paired t-tests. In this example, although an increase in apoptosis after irradiation of the rectal cancer biopsy was identified, this was not found to be significant (p = 0.178).

	Control tis	sue		Irradiated tissue		
Random	DAPI	FITC	Apoptotic	DAPI	FITC	Apoptotic
area	area	area	ratio (%)	area	area	ratio (%)
А	254449	33350	13.1	139649	134399	96.2
В	302872	96350	31.8	171484	68601	40.0
С	395301	194284	49.1	168942	168942	100
D	145768	53292	36.6	143382	48118	33.6
Mean	274597.5	94319	32.7	155864.3	105015	67.5
SD	103877.8	71641.6	14.9	16670.8	56312.6	35.5

Although levels of apoptosis were found to be higher in each of the irradiated murine samples, this was not found to be statistically significant in any of the individually irradiated samples in relation to the control (Figure 46). However, interestingly the level of apoptosis in tissue irradiated with 30Gy was noted be significantly higher than that irradiated with 10Gy (p = 0.03).

This same method of analysis was applied to the human rectal cancer samples.



Figure 46) Bar chart illustrating the apoptotic ratio using the TUNEL assay after irradiation of murine colorectal tissue with 2Gy (n = 2 devices), 10Gy (n = 2 devices) and 30Gy (n = 3 devices) (Standard Error of the Mean shown in error bars). Paired t-test significance values of irradiated tissue compared to control tissue are shown in bold.

3.9.2 TUNEL assay in human rectal cancer tissue

Apoptotic response to radiation of human rectal cancer tissue was seen to be variable, however, in the majority of patient samples interrogated, a trend towards increased apoptotic ratios was seen in nine of the patients (Figure 47). Paired t-tests were again performed using a minimum of three randomly selected areas as described above in section 3.9.1. In two of the patients (patient 7 and patient 10), although the increase in apoptotic ratio appeared to be meaningful, this was not found to be significant (p = 0.0839and p = 0.108 respectively). However a significant increase was identified in one patient sample (patient 5) (p = 0.02) (Figure 48).

a) Non-irradiated control tissue



Figure 47) Representative images of human rectal cancer tissue (n = 9 devices) stained using the TUNEL assay after maintenance within a microfluidic device for greater than 48 hours (x400 magnification). A trend towards increased levels of apoptosis was demonstrated after irradiation with 2Gy.

- a) Non-irradiated tissue
- b) Irradiated tissue exposed to a 2Gy dose after 26 hours of maintenance within the microfluidics chamber
 - i) Blue nuclei DAPI counterstained
 - ii) Green nuclei FITC labelled apoptotic nuclei
 - iii) Composite image


Figure 48) Bar chart summarising changes in levels of apoptosis using the TUNEL assay after irradiation (2Gy) of the eleven patient rectal cancer samples interrogated. (Standard Error of the Mean shown in error bars) Paired t-test significant increases in apoptotic ratio of the irradiated tissue compared to control tissue are shown in bold.

Grouping of patient samples was performed to compare control, nonirradiated samples with irradiated samples in a similar manner to that performed for caspase cleaved cytokeratin as demonstrated in section 3.8. The patient samples were grouped according to the apoptotic ratio in each of the patient irradiated samples. Groups were classified as follows: Low levels of apoptosis <30%, Medium 30-50% and High >50% (Raw data shown in appendix 8). However, only one patient could be classified in this low group.

A mean increase in apoptotic ratio, as demonstrated with use of the TUNEL assay was observed in the irradiated tissue samples when compared to the control samples in the medium release group upon unpaired t-testing (p < 0.0001) (Figure 49). A statistically significant difference was also identified in the high release group (p = 0.028).



Figure 49) Bar chart summarising change in apoptotic ratio after irradiation as assessed using the TUNEL assay amongst the three grouped patient samples interrogated. The patient samples were grouped into low (<30%), medium (30-50%) and high (>50%) levels of apoptosis according to level of apoptotic ratio in each of the patient irradiated samples. (Standard Error of the Mean shown in error bars) Unpaired t-test significant increases in apoptotic ratio of the irradiated tissue compared to control tissue are shown in bold.

An increase in level of apoptosis as deemed by the TUNEL assay was seen to increase in almost all of patient samples irradiated reaching levels of significance upon grouping. However using individual patient analysis, a significant increase in apoptotic ratio was only identified in one patient.

3.10 M30 CytoDeath[™] immunohistochemistry

As seen with the TUNEL assay (section 3.9.2), variable apoptotic response of the rectal cancer biopsies was seen after irradiation, as assessed by the M30 cytotoxicity immunohistochemical assay. A loss of architecture was also seen as previously demonstrated on H&E staining (section 3.7) after irradiation with a loss of cytokeratin stained cells in all irradiated tissue. Despite this loss of morphology, caspase cleaved cytokeratin was identified in these regions, with a trend towards increased apoptotic ratios after irradiation, when compared to the non-irradiated control tissue in the majority of the patients (n = 9) (Figure 50).

Paired t-testing was again performed using a minimum of three randomly selected areas, as described above in section 3.8.1. Although increases in apoptosis were seen in the majority of samples interrogated, these were only found to be significant in four of the eleven patient samples after paired t-testing ($p \le 0.05$) (Figure 51).



Figure 50) Representative images of human rectal cancer tissue (n = 9 patients) stained using the M30 cytotoxicity assay after maintenance within a microfluidic device for greater than 48 hours (upper panel x100 magnification; lower panel x 400 magnification). A trend towards increased levels of apoptosis was demonstrated after irradiation with 2Gy.

- a) Prior to microfluidics
- b) Non-irradiated tissue observed after 49.5 hours
- c) Irradiated tissue (2Gy) observed after 49.5 hours



Figure 51) Bar chart summarising changes in levels of apoptosis using the M30 cytotoxicity assay after irradiation of the eleven patient rectal cancer samples interrogated. (Standard Error of the Mean shown in error bars) Paired t-test significant increases in apoptotic ratio of the irradiated tissue compared to control tissue are shown in bold.

Grouping of patient samples was performed to compare control, nonirradiated samples with irradiated samples. The patient samples were initially grouped according to the mean percentage area undergoing apoptosis in each of the patient irradiated samples. Groups were classified as follows: Low apoptotic ratio <15%, Medium 15-30% and High >30% (Raw data shown in appendix 9).

A mean increase in apoptotic ratio was identified in the irradiated tissue samples when compared to the control samples in the low & medium release groups, however this was not found to be statistically significant upon unpaired t-testing (p = 0.1117 & p = 0.2003 respectively). (Figure 52) In the high release group, this increase after irradiation was found to be statistically significant (p < 0.0001).



Figure 52) Bar chart summarising change in apoptotic ratio as assessed using M30 cytotoxicity assay amongst the three grouped patient samples interrogated. The patient samples were grouped into low (<15%), medium (15-30%) and high (<30%) levels of apoptosis according to the level of apoptotic ratio in each of the patient irradiated samples. (Standard Error of the Mean shown in error bars) Unpaired t-test significant increases in apoptotic ratio of the irradiated tissue compared to control tissue are shown in bold.

Although an increase in level of apoptosis as deemed by the M30 cytotoxicity assay was seen to increase in almost all of patient samples irradiated, levels of significance were only observed in the high release group. Upon use of individual patient analysis, levels of significance (p < 0.05) were identified in four of the eleven patients.

It is worth noting however, that the apoptotic ratios measured using the immunohistochemical M30 cytotoxicity assay were lower than those when assessed using TUNEL assay.

3.11 Metabolomic analysis

Overnight collections of effluent were used for metabolomics analysis with comparisons made between control and irradiated samples and alterations in metabolite release with time. The incubation times of each sample are demonstrated in table 21.

Table 21) Table to demonstrate the effluents used for metabolomic	S
analysis	

Patient	Incubation time of	Total period	Incubation time of	Total period
	control sample and	of collection	control sample and	of collection
	sample prior to	(hours)	sample post to	(hours)
	irradiation (hours)		irradiation (hours)	
1	6-21	15	33-44	11
2	6-19	13	31-43	12
3	6-22	16	34-46	12
4	6-22	16	32-45	13
5	6-21	15	33-45	12
6	6-19	13	31-42	11
7	6-18	12	30-41.5	11.5
8	8-23	15	31-47	16
9	6-21.5	15.5	31.5-46	14.5
10	6-22.25	16.5	32.25-46.25	14
11	6-20.5	14.5	28.75-44.75	16

Initial metabolomic analysis was used to assess the effluent collected from the irradiated tissue prior to irradiation and post-irradiation in patient samples. All effluent prior to irradiation along with technical replicates were loaded into the pre-irradiation class and compared with all the postirradiation samples and technical replicates. This comparison revealed 28 compounds that allowed the two classes to be differentiated (p < 0.0001), with these compounds being increased in the post-irradiation samples. These compounds are shown in appendix 10)A. Principal component analysis of the effluent collected between the two time periods in the irradiated sample is shown in Figure 53.



Figure 53) Principal component analysis of compounds from irradiated tissue in effluent collected prior to irradiation and post irradiation demonstrating differentially expressed compounds (p < 0.0001).

In a similar manner, a comparison between the effluents collected at the corresponding two time periods in the control specimen was performed. This identified 31 differentially expressed compounds (p < 0.0001) (Figure 54).



Figure 54) Principal component analysis of compounds from control tissue in effluent collected at corresponding time periods prior to the irradiated sample being irradiation (control 1) and post irradiation (control 2) demonstrating differentially expressed compounds (p < 0.0001).

Finally, the effluent from the post irradiation sample was compared to the corresponding time dependent control. There were only three compounds identified that distinguished the two, which would suggest that the

difference seen between the effluent prior to irradiation and postirradiation is one that is time-dependent, rather than due to the effect of radiation itself. These compounds are also shown in appendix 10)B. Principal component analysis of the effluent collected between the effluent collected post-irradiation in the irradiated sample and the corresponding control is shown in Figure 55.



Figure 55) Principal component analysis of compounds from irradiated tissue in effluent collected post irradiation and its time comparative control demonstrating differentially expressed compounds (p < 0.0001).

It was noted that to perform this analysis in greater detail, the experiment may require samples of greater concentration or volume in order to identify potential compounds that may determine radiosensitivity and/or radioresistance.

3.12 Clinical correlation

It was anticipated that correlation of laboratory response with clinical response would be performed. However, due to the limited number of patient samples (n = 11) and with only five of these patients with resectional histology after long course radiotherapy available, this was not deemed possible. None of the patients achieved a complete pathological response and therefore it was not possible to make a correlation with the outcomes of the assays used in this study or identify which of these is most

sensitive.

Despite the multiple methodologies used to assess radiosensitivity and/or radioresistance, there appeared to be little overlap in their findings. Although one patient was shown to have a significant increase in levels of apoptosis as assessed using TUNEL assay, this did not correlate with the patients thought to be potentially responsive based on caspase cleaved cytokeratin release, as assessed using the LDH cytotoxicity assay, or the M30 Cytotoxicity ELISA, or immunohistochemically using the M30 Cytotoxicity assay. A summary of these findings correlated with clinical response is demonstrated in table 22. **Table 22)** Table to summarise the clinical outcome of all eleven patients in the study correlated with potential biomarkers of responseto irradiation. (TRG – tumour regression grade)

Patient	Mandard	Response of irradiated tissue (<i>ex vivo</i>)					
	TRG	LDH cytotoxicity assay	M30 ELISA	TUNEL assay	M30 immunohistochemical cytotoxicity assay		
1	N/A	No increase (p = 0.50)	No increase (p = 0.10)	Trend towards increase (p = 0.17)	Trend towards increase (<i>p</i> = 0.16)		
2	N/A	Trend towards increase the following day ($p = 0.09$)	Significant increase (p = 0.04)	Trend towards increase (p = 0.20)	No increase (p = 0.65)		
3	N/A	Trend towards increase within 4 hours of irradiation ($p = 0.85$)	No increase (p = 0.09)	Trend towards increase (<i>p</i> = 0.14)	Trend towards increase (<i>p</i> = 0.76)		
4	TRG 4	Trend towards increase the following day (<i>p</i> = 0.65)	Significant increase (p = 0.05)	Trend towards increase (p = 0.93)	Significant increase (p = 0.04)		
5	TRG4	No increase (p = 0.20)	No increase (p = 0.08)	Significant increase (p = 0.02)	Trend towards increase $(p = 0.27)$		
6	TRG4	Trend towards increase within 4 hours of irradiation ($p = 0.07$)	No increase (p = 0.09)	<i>No Increase</i> (<i>p</i> = 0.30)	Significant increase (p = 0.03)		
7	N/A	No increase (p = 0.50)	Significant increase (p = 0.01)	Trend towards increase (p = 0.08)	Trend towards increase (p = 0.41)		
8	N/A	Trend towards increase within 4 hours of irradiation ($p = 0.20$)	No increase (p = 0.38)	Trend towards increase (p = 0.18)	Significant increase (p = 0.01)		
9	TRG 3/4	Trend towards increase within 4 hours of irradiation ($p = 0.10$)	No increase (p = 0.08)	Trend towards increase (p = 0.50)	No increase (p = 0.09)		
10	TRG4	Significant increase the following day (p = 0.03)	Not calculated due to anomalous result in control	Trend towards increase (p = 0.11)	Significant increase (p = 0.04)		
11	N/A	No increase (p = 0.24)	Significant increase (p = 0.04)	Trend towards increase (p = 0.06)	Trend towards increase (p = 0.23)		

SECTION 4: Discussion

4.1 LDH as a biomarker

4.1.1 Assessment of tissue viability after maintenance in a microfluidic device

LDH is a stable cytoplasmic enzyme that is present in all cells and is released when plasma membranes are damaged.(390,391) Hattersley *et al* have previously demonstrated LDH to act as suitable marker of cell death in HNSCCs after the infusion of chemotherapeutics and therefore, its role was also investigated in this study.(380)

Measured LDH levels of the effluent were always high within the first few hours of being placed within the microfluidic device and fell reproducibly to almost negligible levels (<50units/g wet weight of tissue) within a median time of 28 hours, in both murine colorectal tissue and human rectal biopsies. This trend had previously been identified in the maintenance of murine liver tissue and HNSCC biopsies.(378–381,416) These initial high levels of LDH release have been attributed to the high levels of cell death involved in tissue preparation and set up within the microfluidic device; a similar argument seemed appropriate from this study too. After this initial period there was stabilisation in LDH levels, confirming that flow of the supplemented media through device did not lead to further cellular injury. Both murine colorectal tissue and human rectal cancer tissue were maintained for over 70 hours within the microfluidic device. Evidence of viability was determined by increased LDH release after a combination of mechanical and chemical lysis and preservation of morphology after H&E staining. It was also noted that throughout these periods of maintenance of tissue within the microfluidic devices, tissue architecture was maintained centrally, confirming the diffusion of nutrients and waste products through the tissue.

This period of time is similar to that previously achieved by Webster *et al* in the maintenance of colorectal tissue.(376) Carr *et al* has previously demonstrated maintenance of murine liver tissue for periods of up to 341 hours, with evidence of tissue viability based on LDH release.(381) Current

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attempts to maintain murine colorectal and rectal cancer tissue samples for longer periods were not successful however, due to tissue disintegration beyond 70 hours. Despite this, LDH surges were not detected. This finding is hypothesised to be tissue specific.

Attempted chemical lysis of murine colorectal tissue and human rectal biopsies with various concentrations of the lysis solution obtained from the Cytotoxicity LDH Kit^{PLUS} kit[™]or Triton X-100 did not induce effective rupture of the cells, as evidenced by the lack of increase in LDH release, in addition to partly retained crypt architecture on H&E staining. LDH release was only effectively induced with the addition of mechanical lysis, which would suggest a resistance of both murine colorectal and human rectal tissue to these chemicals. This data contradicted work performed with HNSCC and murine liver tissue, where introduction of a lysis agent induced cell death.(378–381)

Colorectal cancer commences superficially in the mucosa before spread into deeper layers.(2,417,418) The majority of diagnostic biopsies taken from rectal tumours are from the mucosa and submucosa.(419) This mucosa has several mechanisms that protect the tissue from insult. Mucosal epithelial cells are continually sloughed and replaced as part of the normal regenerative process.(417,420) The epithelium is formed by cells joined by tight junctions, which act as a physical barrier to both micro-organisms and macro-molecules, but permit the diffusion of ions and water.(421) There are also multiple cell types that form the epithelium that aid in this protective role, including goblet cells which synthesise and secrete mucin, which acts as protective mucus blanket.(422) M-cells initiate mucosal immune responses and involve the transportation of antigens and micro-organisms to the underlying lymphoid tissue.(423)

HNSCCs originate from the epithelial squamous cells that line head and neck mucosal surfaces, unlike adenocarcinomas which arise from glandular tissue.(424,425) As with colorectal tissue, there is continual regeneration of cells, along with several mucosal protective mechanisms, however, not all of the protective cells that are present in colorectal mucosa are present in HNSCC tissue. Rectal mucosa is also noted to be less permeable to higher

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molecular weight molecules than mucosa of the head and neck in the delivery of therapeutic drugs.(426–428) In addition, murine liver tissue also demonstrated increased LDH release after addition of the lysis agent, but this is again likely related to the high permeability associated with hepatocytes to permit the diffusion of multiple molecules involved in metabolism and detoxification.(429,430)

Therefore it is hypothesised that it is a combination of mechanical, cellular and chemical protective measures present in colorectal epithelium that provide an effective barrier against the toxic nature of the lysis agent and Triton-X-100 on the mucosal surface of the biopsies, in the same manner as within the colon during the passage of toxic components, including ammonia and bacteria that are present for relatively long periods of time.(431–433)

As per previous groups, both murine colorectal tissue and rectal cancer biopsies were successfully maintained within the microfluidic devices after previous cryopreservation, with no evidence of loss of viability despite this.(379–381)

4.1.2 LDH response to irradiation

This study is the first to investigate the effects of radiotherapy on colorectal tissue maintained using a microfluidic device. Prior to this, only Carr *et al* have used the platform to study the effects of radiotherapy to maintain murine liver tissue and HNSCC samples.(381)

Irradiated murine colorectal and human rectal cancer tissue reproducibly lost architectural integrity, with loss of well-defined crypt structures evident on H&E staining at all radiation doses (2Gy, 10Gy and 30Gy). This was not noticeably different between increasing levels of radiation exposure in the murine samples. However, LDH levels were not markedly increased upon irradiation of the murine tissue with 2Gy and 10Gy doses, despite tissue damage. High levels of LDH release were only induced by very high levels of radiation (30Gy) and these were shown to occur within hours of exposure. These findings were also confirmed by Carr *et al*, who also noted significant LDH surges after irradiation with single fractions of 20Gy doses in murine liver tissue and 40Gy in HNSCC tissue.(381) In Cai's study of primary human central nervous system cultures, only exposure to very high doses (60Gy) caused a marked rise in LDH release. (434) Despite not identifying increases in LDH at lower doses (\leq 30Gy), the authors noted evidence of DNA fragmentation as marker of apoptosis. Rao's study of HeLa cell cultures also described immediate rises in LDH after irradiation, however, these were noted to occur after much lower doses of radiation (3Gy).(435) Central nervous system cells are noted to have a lower sensitivity due to their relatively low mitotic frequency than the cervical cells that HeLa cells are derived from, which would explain the discrepancy between Cai and Rao's findings.(436,437) Spheroids have been demonstrated to have a greater radioresistance than cell lines grown as monolayers, which was hypothesised to be due to the intercellular communication and a contact effect through the exchange of substances related to DNA repair. (438–441) It is also likely for these reasons that the tissue biopsies used in this study did not elicit the same LDH response to low doses of irradiation demonstrated by Rao.

LDH has been shown to be a marker of necrosis and does not necessarily distinguish whether this is due to primary necrosis, or secondary to apoptosis.(442) In addition, use of the Cytotoxicity LDH ^{PLUS} kit[™] has been suggested to underestimate the number of dead cells in the presence of growth inhibition, which is a known consequence of radiotherapy.(443,444) Other mechanisms of radiation induced cell death such as apoptosis, mitotic catastrophe, senescence and autophagy may not be accounted for and therefore LDH release if induced, may not be immediately observed.(445– 448)

Although LDH has been used to assess response to radiation in multiple other organs, there are very few studies that have reported the role of LDH as a specific marker of response to radiation in rectal cancer.(449–452) Nakazawa *et al* subjected human colonic adenocarcinoma cell lines grown as a three-dimensional culture to a single 90Gy dose of radiation and noted LDH levels to increase immediately after irradiation, with a peak over four times that of the controls, which occurred at ten days.(453) Patterns of LDH release were also noted to correlate with the number of non-viable tumour cells. Although lower doses (5Gy, 10Gy and 15Gy) were investigated, they were unable identify significant differences in LDH levels between irradiated cell lines and controls. The authors noted that LDH was an "insensitive dose-response" indicator "and that at low dose levels (i.e. those relevant in radiation therapy), they would not demonstrate statistically significant responses", supporting the findings of my study. Pompecki et al reported a significant increase in serum LDH in patients with colorectal tumours, when compared to control patients with peaks in activity demonstrated four days after irradiation.(454) Unfortunately, due to this paper being published in German with no English translation available, whether response can be predicted according to LDH levels has not been identified. Buijsen *et al* used the pre-therapeutic blood samples of patients undergoing LCCRT, but were unable to identify any difference in value according to pathological response. (455) Fahmueller et al used pre-therapy serum LDH to assess response of colorectal malignancies with liver metastases to selective internal radiation therapy and correlated low levels with responders (p = 0.011).(456)

In all of these studies, radiation has been delivered as a single dose and it is yet to be determined whether LDH could act as a sensitive marker of response to fractionated therapy. Rave-Fränk *et al* exposed rats to selective liver fractionated irradiation (thirty fractions of 2Gy) or high dose irradiation (single fraction of 25Gy).(457) Although serum LDH was found to increase after high dose irradiation, this increase was not seen in the fractionated cohort. Carr *et al* delivered fractionated doses of radiation (5 x 2Gy) to rat liver and HNSCC biopsies, but did not identify notable increases LDH.(381) Saito *et al* exposed xenografts of two cells lines, SCC VII (murine SCC cell line) and HT-29 (colonic cancer cell line) to three fractions of 10Gy and noted LDH activity to be lower in the irradiated SCC VII tumours when compared to control, non, irradiated tumours.(458) This finding however was not replicated in the colonic cell line. *In vivo* external beam radiotherapy is usually delivered as approximately five fractions of 5Gy in SCRT or five fractions of 1.8Gy per week for five weeks in the UK.(118) Given the lack of meaningful increases in LDH release after irradiation in this study, it was deemed that LDH would unlikely prove to be a potential biomarker of radiosensitivity at clinically relevant doses. Although the effect of fractionated doses of radiation has not been evaluated in this study, based on the work of several others, it is unlikely that LDH would prove to be a sensitive biomarker, even in this setting.

4.2 M30 Cytodeath[™] ELISA as a biomarker

Caspases cleave various cellular proteins including keratin 18 during apoptosis and exposure of the M30-neo-epitope is recognised by the M30 antibody.(394,395) Hägg *et al* have previously assessed apoptosis, using M30 ELISA to screen various chemotherapeutics on human breast cell carcinoma cell lines and noted that this activity correlated with annexin V, another marker of apoptosis.(459)

In this study, initial caspase cleaved cytokeratin release was demonstrated to be high within the first few hours of the tissue sample being placed within a microfluidic device in the six patient samples, where early time points were evaluated. Levels then decreased to negligible levels, resembling the pattern of release of LDH release. This again is likely due to initial cellular injury, where caspase cleaved cytokeratin is immediately released into the media perfusing the tissue biopsy.

Overall two patterns of activity were observed in assessing the release of caspase cleaved cytokeratin after irradiation. In the first group, seven of the eleven human tissue samples analysed demonstrated no increase in apoptosis after irradiation, whereas the second group containing the remaining four patient samples, demonstrated increased levels of apoptosis within two hours of irradiation. Analysis of these patient tumours individually, where there were significant increases in caspase cleaved cytokeratin release after irradiation in four of the patients, may potentially be an indicator of response to radiotherapy *in vivo*. Unfortunately in the

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patients with elevated levels of caspase cleaved cytokeratin in response to irradiation, only one patient underwent LCCRT, with subsequent pelvic exenteration due to the advanced nature of the tumour at resection. The histopathology of the resected specimen demonstrated an absence of regressive changes (Modified Mandard TRG 4). Given these limited results, clinical outcome could not be correlated with M30 ELISA outcome. Upon grouping of patient samples to compare non-irradiated, control samples with irradiated samples, a mean increase in caspase cleaved cytokeratin was only observed in the irradiated tissue samples when compared to the control samples in the low release group, however, this did not reach significance (p = 0.0613). In this grouped analysis, it is worth noting that this method of evaluation fails to take into account individual patient tumour heterogeneity including potential radiosensitivity and resistance, which may account for these findings. Despite these results, the findings in the low concentration group would suggest that irradiation does lead to an increase in caspase cleaved cytokeratin as measured using M30 ELISA.

Hägg's study also involved assessment of intracellular caspase cleaved cytokeratin, in addition to that released, by adding a non-ionic detergent to the tissue culture medium.(459) This would suggest that M30 ELISA may therefore not fully account for all apoptosis, as not all caspase cleaved cytokeratin is liberated immediately from the cells.

There does not appear to be any work conducted with the use of M30 ELISA in assessing response to radiation, which is likely due it being a relatively new assay. Several authors have used the M30 ELISA to evaluate response to chemotherapy in a variety of cancers, but none of these have been able to establish its use as a predictive tool. Ausch *et al* quantified serum M30 levels around the delivery of chemotherapy in patients with colon cancer.(460) Concentrations of M30 were noted to be significantly higher in cancer patients than healthy controls, but correlations with response to chemotherapy were not found.

Several studies have also used M65 ELISA in addition to M30 to assess cell death, which measures the levels of caspase cleaved and intact cytokeratin

18, as a method of differentiating apoptosis and necrosis.(383,461,462) The M65 assay measures total cell death, necrosis and apoptosis; therefore when used in association with the M30 ELISA, can quantify the relative contribution of apoptosis and necrosis to total cell death. Kramer *et al* noted that in addition to release of caspase-cleaved cytokeratin from tumour cells during apoptosis, in the event of cellular necrosis, there is also additional release of soluble cytokeratin, which can be identified using the M65 ELISA.(383)

Oven Ustaalioglu et al's study evaluated serum values of M30 and M65 in patients with advanced non-small cell lung cancer, but were unable to identify any correlation between M30 value and progression-free survival.(463) However, M65 appeared to be predictive in this study. Bilici et al evaluated serum levels of M30 and M65 before and after chemotherapy for advanced gastric cancer. (464) Levels of both were significantly increased in post-therapeutic samples when compared to pre-therapeutic levels. Patients with lower increases after therapy were found to have better median progression-free survival and overall survival. However, on logistic regression analysis, only alteration in M65 after chemotherapy was found to be an independent factor in predicting response. It must be noted that in clinical practice, prediction based on a post-therapy sample is not practical. Yildiz et al reviewed pre-chemotherapeutic levels of M30 and M65 in patients with ovarian cancer and although both markers were significantly elevated when compared to healthy controls, only M65 levels appeared to be predictive of resistance to chemotherapy (p = 0.04).(465) Tas *et al* assessed levels of serum M30 in patients prior to chemotherapy in patients with melanoma, but were unable to find any association of baseline levels with chemotherapeutic response. (466) The group subsequently evaluated serum levels of M30 of patients with breast cancer.(467) Although levels were found to be significantly higher in patients with metastatic than localised disease, no correlation was seen with response to chemotherapy. Due to the lack of work exploring M30 ELISA as a marker of response to radiation, further studies evaluating its role are required. Based on work carried out assessing its role in chemotherapy, it may not be a successful

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indicator of outcome. However the role of M65 in conjunction with M30 should be considered in future studies.

4.3 TUNEL as a biomarker

The assay was used to label free 3'-OH terminal DNA strand breaks with fluorescein-deoxyuridine triphosphate (dUTP) modified nucleotides to identify and quantify apoptosis at a single cell level.(400) Although previous authors have performed quantitative analysis by calculating individual apoptotic cells, this method was not used as it would not have appropriately taken into consideration the coalescence of individual apoptotic bodies.(412–414) Therefore to avoid under-calculation of the apoptotic ratio, the areas occupied by DAPI counterstaining and the FITC labelling (apoptotic nuclei) were used.

Although irradiation of murine colorectal tissue with single fractions (2Gy, 10Gy or 30Gy) demonstrated a trend towards increased levels of apoptosis, this was not found to be statistically significant. Analysis of murine colorectal tissue involved a limited number of irradiated samples and therefore it is likely that statistical significance was not achieved due to the small sample size of each group.

In the majority of patient samples (n = 9 patients) interrogated, a trend towards increased apoptotic ratios was seen, with three of these patients appearing to have meaningful increases. Although a significant increase was identified in one patient sample (p = 0.02), this did not appear to correlate with clinical outcome, despite undergoing LCCRT with subsequent APER, with the histopathology of the resected specimen demonstrating an absence of regressive changes (Modified Mandard TRG 4).

Upon grouping of patient samples to compare control, non-irradiated samples with irradiated samples, a mean increase in apoptosis was demonstrated with the TUNEL assay in the irradiated tissue samples when compared to the control samples in the medium (p < 0.0001) and high release (p = 0.0281) groups. This was not identified in the low release group, but this may be due to the use of only one patient in this group. As discussed above in section 4.2, grouped analysis does fail to take into account tumour

heterogeneity between patients. However, these findings would suggest that irradiation does lead to an increase in apoptosis as measured by TUNEL in both murine colorectal and human rectal cancer tissue.

TUNEL has been used in multiple studies to identify apoptosis in colorectal cancers.(413,468,469) There has been limited work performed to assess the role of TUNEL to evaluate apoptosis after irradiation of rectal tissue, with studies either assessing levels in pre-therapeutic biopsies or resectional tissue. Sakura *et al* assessed rectal tumours (n = 16) that had been exposed to hyperthermic chemoradiotherapy and examined TUNEL positivity in the resected tumour cells.(470) Very few apoptotic cells were noted in the non-irradiated patients, when compared to the treated patients (p < 0.05). In the treated group, apoptosis was correlated to be significantly higher, according to improved pathological outcome.

McDowell *et al* identified increased apoptotic indices in the pre-treatment biopsies of rectal cancer to correlate with improved TRG after to LCCRT (p = 0.0051).(471) However, Huerta *et al* were not able to identify a significant difference in apoptosis in relation to pathological response after neoadjuvant therapy (p = 0.470).(262) Liu *et al* evaluated eighty patients, with half (n = 40) undergoing neo-adjuvant radiotherapy prior to resectional surgery and the other half undergoing surgery without preceding treatment.(472) The apoptotic index was significantly higher in the resectional tissue after radiotherapy than in the pre-treatment biopsies (p = 0.013). However, it is not known whether this also led to improved clinical outcomes.

Although there is limited work involving rectal cancer, nick-end labelling has been used to assess response to radiation to a variety of other cell lines and human tissue. Lee *et al* investigated the effect of exposure to radiation (2Gy) on the model cell lines, U937 (human leukemic monocyte lymphoma cell line) and HeLa. After irradiation, DNA fragmentation was induced as identified using TUNEL.

Ohno *et al* assessed irradiated cervical tumours (SCCs) before and after irradiation (five fractions of 1.8Gy) for nick-end labelling using ApopTag[™], a method of detecting single and double stranded DNA breaks.(473)

ApopTag[®] has been shown to differentiate between apoptotic and necrotic cell death.(474) A significant increase was noted in apoptotic index between pre- and post- therapeutic biopsies (p = 0.0004). Bhosle *et al* also assessed cervical malignancies and used TUNEL to assess apoptotic index as predictive marker for radiosensitivity of cervical carcinoma after the first dose of fractionated radiotherapy (2Gy).(475) A very highly significant correlation was identified between changes in apoptotic index and tumour regression following radiotherapy, with increase in apoptotic index found to be significant in patients classed as complete responders (p < 0.001), but not in partial responders (p = 0.07).

Su *et al* assessed the effects of radiation on Epiderm, 3D human skin constructs and used TUNEL to identify apoptotic cells.(476) Apoptosis was shown to be higher after irradiation with doses of 2.5Gy and 5Gy, than in the non-irradiated control at the two time points assessed, 24 and 48 hours. This also confirms our findings of apoptosis being detectable after 24 hours of irradiation. Overall these studies would suggest a potential for prediction of radiosensitivity based apoptotic response.

The lack of correlation between results obtained using of M30 caspase cleaved cytokeratin and TUNEL as markers of apoptosis may be explained due to the cleavage of cytokeratin occurring earlier in the apoptotic cascade prior to DNA nick-end labelling.(383) Several authors have reported concerns over false-positive staining in addition to high background staining and also staining for necrosis. (412,477–481) False-positive staining has been attributed to several reasons: firstly non-specific staining can occur as a result of initial fixation of the tissue that is either too extensive, incomplete or delayed; secondly, artificial strand breaks can be induced by 'unmasking' after formalin fixation and paraffin embedding, and therefore it is also possible that this may occur as a result of the permeabilisation solution in the protocol used.(413) Necrosis has been shown to also result in DNA strand breaks and therefore would also stain positively.(477,482,483) Given these concerns and our own findings, we would also advocate that apoptosis is assessed using multiple techniques, such as M30 rather than using TUNEL as a sole modality.

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4.4 M30 Cytotodeath[™] immunohistochemistry as a biomarker

A variable apoptotic response of the rectal cancer biopsies was seen after irradiation as assessed by the M30 cytotoxicity immunohistochemical assay, however, losses of architecture and a loss of cytokeratin stained cells identified were seen in all irradiated specimens. Caspase cleaved cytokeratin was identified in these regions despite this insult and a trend towards increased apoptotic ratios after irradiation when compared to the non-irradiated control tissue was identified in the majority of the patients. These increases were found to be significant in only four of the eleven patient samples ($p \le 0.05$).

Significant increases in caspase cleaved cytokeratin release after irradiation in individual patients was assessed to identify if this was a potential indicator of clinical response. Of the four patients with elevated levels of caspase cleaved cytokeratin in response to irradiation, although all four underwent long course radiotherapy with or without neo-adjuvant chemotherapy, only three of these survived to undergo resectional surgery. In these three patients modified Mandard grading demonstrated an absence of regressive changes in all of the resected specimens (TRG 4). As demonstrated in section 4.3 with the use of TUNEL, grouping of patient samples to compare control, non-irradiated samples with irradiated samples, demonstrated a mean increase in apoptotic ratio in the irradiated tissue samples in the high release group (p < 0.0001). Although grouped analysis fails to take into account individual patient tumour heterogeneity, the findings would confirm that irradiation does lead to an increase in caspase cleaved cytokeratin as measured using M30 immunohistochemistry. As described previously in section 3.10, apoptotic ratios measured using the immunohistochemical M30 cytotoxicity assay were lower than those when assessed using TUNEL assay, which may be attributable to the overscoring discussed in section 4.3. As discussed in section 4.2, Hägg et al suggested that M30 ELISA may not evaluate apoptosis entirely, as not all caspase cleaved cytokeratin is liberated immediately from the cells, which would

account for the discrepancy in findings between the two techniques of ELISA and immunohistochemical analysis in this study.(459) It should also be noted that apoptosis can also occur independently of caspase dependent pathways and therefore M30 would fail to take into account these mechanisms.(484–486) The combination of possible over-scoring by TUNEL and underscoring using both of the M30 analysis techniques that may be responsible for the lack of correlation between these methods of characterising apoptosis.

Mirzaie-Joniani *et al* have previously shown apoptosis to be induced after irradiation (5Gy) of HeLa-Hep2 cells using TUNEL and M30 immunohistochemistry. Although irradiation with 2Gy did not induce significant increases in apoptosis as assessed with TUNEL, use of M30 identified the presence of significant apoptosis (p < 0.04). The authors attributed this variability in detection due to M30 detecting an earlier stage of apoptosis, when compared to TUNEL.(395,481,487) In their paper, they describe M30 detection of apoptosis to be higher than that using TUNEL, which would contradict the findings in this study and the suggestion of overscoring using TUNEL described in section 4.3.

As with TUNEL, there is limited published data assessing the role of M30 immunohistochemistry to evaluate apoptosis after irradiation of rectal tissue, with these studies either assessing levels in pre-therapeutic biopsies, or resectional tissue.

De Bruin *et al* reviewed levels of apoptosis using immunohistochemical detection of M30 by using tissue microarrays of a large number of tumour samples (n = 1067: 560 non-irradiated and 507 irradiated) from the Dutch Total Mesorectal Excision trial.(488) Median levels of apoptosis were noted to be higher in irradiated tumours than those that were not irradiated (p < 0.001). Although a correlation between low intrinsic apoptosis with local recurrence was observed, radiation-induced apoptosis was not found to correlate in the same manner. Subsequently, Gosens *et al* used tissue microarrays to assess immunohistochemical expression of M30 to quantify apoptosis, before and after neo-adjuvant chemoradiotherapy.(489)

specimens when compared with the pre-therapeutic biopsies (p < 0.001). Greater levels of apoptosis were noted in the post-therapy specimens in patients with limited tumour regression (p = 0.003), however, pretreatment levels were not found to be predictive.

Saigusa *et al* performed M30 immunostaining of resectional rectal tissue after neo-adjuvant chemoradiotherapy and identified 34 patients (47%) with M30 positive tumours.(490) A significant correlation was observed between patients without M30 staining and advanced disease (p = 0.011) and tumour recurrence (p = 0.038), however, no correlation with TRG was noted. Saigusa's findings contradict that of others, which the authors attribute to the fact that the apoptosis noted in these resectional specimens represents spontaneous activity rather than that secondary to neo-adjuvant therapy due to the interval between neo-adjuvant therapy and surgery, with apoptotic cells likely to have undergone subsequent fibrosis.

Although limited, these studies would confirm the findings in this study of radiation inducing apoptosis as assessed using M30 immunohistochemistry, but its role of as a marker of response to radiation is one that requires further evaluation.

4.5 Metabolomic analysis to identify biomarkers

Although 28 compounds were identified that significantly differentiated (*p* < 0.0001) effluent collected from tissue prior to irradiation and after irradiation, further evaluation would suggest that this difference is time-dependent, rather than an effect attributable to radiation. Due to the limited number of samples available for analysis and limited data with which to perform clinical correlation, metabolomic analysis was unable to be used to identify any potential compounds that could determine radiosensitivity or resistance.

It was noted that to perform this analysis in greater detail, the experiment may require samples of greater concentration or volume in order to identify potential compounds that may determine radiosensitivity and/or radioresistance. This study only analysed metabolites in effluent samples taken during overnight collections prior to and after radiation exposure and therefore any metabolite release outside these time periods may not be accounted for.

Ghosh *et al* evaluated the metabolic changes in gastrointestinal tissue of mice after exposure to radiation (4Gy or 8Gy) and identified markers correlating with tissue injury using homogenised tissue samples.(491) Their study was able to define nine putative biomarkers of radiation induced gastro-intestinal injury that demonstrated a dose and time-dependent response. Ghosh's study used homogenised tissue samples for assessment of metabolites and this method should also be considered for future work to identify endogenous compounds that are not released into the perfusing media. It is important to recognise that radiation, even at lower doses causes damage to healthy tissue in addition to any therapeutic effect on malignant tissue and therefore any future distinguishing metabolites need validation with in vivo response, to assess if these differences are due to radiation response or potential toxicity. However, use of metabolomics with the microfluidic platform in this study, could act as a platform for prediction of radiation toxicity, in addition to response to neo-adjuvant therapy. Jang *et al* recently performed metabolite profiling on multiple organs including the small bowel (jejunum) of rats exposed to whole body ionising radiation (2Gy or 6Gy).(492) At 24, 48 or 72 after irradiation, the animals were killed and tissue was harvested for subsequent metabolic profiling with nuclear magnetic resonance spectroscopy. They noted multiple significant changes in the high dose (6Gy) group compared with the low dose (2Gy) group. After 48 hours, alterations in eight metabolites were identified to be significant (p < 0.05) between the low dose group and the control, non-irradiated group. These metabolites related to inflammatory response and oxidative stress and were again noted to be dose and timedependent. In jejunal tissue, several of the metabolites were not significantly expressed with respect to the control tissue until 28-72 hours after irradiation to 2Gy. Therefore, it is possible that in this study, differentially expressed metabolites have not been identified due to the insufficient time period of collection of effluent following irradiation.

Relatively recently, Kim *et al* used pre-treatment blood samples (n = 73 patients) to determine response of locally advanced rectal cancer to LCCRT.(493) Initially nine compounds were selected as candidate metabolites. Using the Human Metabolome Database, three known metabolites, hypoxanthine, xanthine and phosphoenolpyruvic acid were identified in the serum as potential markers of response. Higher levels of expression of hypoxanthine and its oxidative product xanthine were identified in patients exhibiting higher tumour regression (p = 0.024). Phosphoenolpyruvic acid levels in contrast were noted to be lower in patients with improved TRG (p = 0.012). Their study confirmed the potential of metabolomics as predictive tool, which requires further validation. Although my study did not identify potential metabolites that were differentially expressed after irradiation, this may be due to the relative radioresistance of the biopsies used as demonstrated by response *in vivo*. Further work with a much greater number of specimens of varying response is required to assess whether microfluidics can be used in this manner. Although limited outcomes have been reported using metabolomics to investigate rectal cancer response to radiation, metabolite expression in response in a variety of other cancers has been assessed. (494-497) Lyng et *al* used cervical carcinoma SCC biopsies (n = 22 patients; n = 44 biopsies) taken prior to and after the first week of radiotherapy (five fractions of 2Gy) and identified a significant correlation between apoptotic cell density, as defined using ApopTag[®] and their standard pulse-acquired spectral profile of lipids. (494) Tumour cell fraction and density were also correlated with differentially expressed metabolites. The authors chose to evaluate apoptosis as a marker of response to radiation. Although the study did not assess whether this translated to improved clinical outcome, their findings demonstrate the potential to use metabolomics as a predictive tool. Wibom et al's study of glioblastomas sampled intracranial extracellular fluid before and during conventional radiotherapy and observed distinct differences between metabolic changes induced by irradiation. (495) Although correlation with response was not performed, the study affirmed that fluid perfusing irradiated tissue could be used to identify distinguishing

metabolites released, which would support future use of the collected effluent for further analysis using microfluidics.

He *et al* used nude mice xenograft models of human pancreatic cancer and exposed them to a variety of radiation doses (10Gy, 20Gy or 30Gy).(496) Tumour tissue sections harvested two weeks later were used for metabolite analysis. Metabolic profiles were not significantly altered in any of the irradiated groups when compared with the control, non-irradiated group, despite significant decrease in tumour volume.

Despite the findings of this current study, metabolomics has a potential to be effective in the identification of future biomarkers of response to radiotherapy and with further developments of this microfluidic platform may allow the delivery of personalised therapies based on outcomes *in vivo*.(498,499)

4.6 Clinical correlation with assessed biomarkers

It has not been determined which of the biomarkers assessed is superior in the prediction of response to radiotherapy for the reasons outlined previously. Based on the findings of this study, LDH is unlikely to function as a potential marker of radiosensitivity, however, the other techniques used may be superior when used in conjunction with each other as a predictive tool.

As discussed in section 1.13, although there is a vast quantity of work assessing the role of potential biomarkers to predict the response of locally advanced rectal cancer to neo-adjuvant therapy, none of this data has been validated. Almost all of the biomarkers that have been identified to date have been identified using retrospective studies without further independent validation. Based on the work carried out by several others investigating potential biomarkers of response to neo-adjuvant therapy, prediction may require a panel of markers to be assessed in parallel to create a model of prediction.(230,262,269)

The TransValid-KF0179/German Rectal Cancer Study Group-Trial was established to prospectively validate previously identified molecular and

clinical biomarkers and hopefully should yield answers when this work is complete. When biomarkers are successfully identified, the treatment of locally advanced rectal cancer would be revolutionised, with patients demonstrated to be responsive to neo-adjuvant therapy *ex vivo* being managed clinically in the same manner and avoiding subsequent significant surgery. Conversely, patients shown to exhibit minimal response *ex vivo* would avoid the potential adverse effects of chemoradiotherapy and could continue to surgery without unnecessary delay.

4.8 Limitations and future work

4.8.1 Patient recruitment, study design and statistical analysis

Although the number of patients recruited to the study was lower than initially anticipated, as this was a pilot study, I was still able to evaluate feasibility, time and in addition provide an insight into potential future work that can be conducted using this platform.

As discovered through this study, only a limited proportion of patients with rectal cancer present through two week wait clinics. Therefore to increase recruitment to future studies, recruitment of patients should be considered through other sources, such as routine outpatient clinics and the NHS Bowel Cancer Screening Programme. However, the potential limitation in recruiting patients from these sources is that the majority of patients would not be identified to have a rectal malignancy prior to endoscopy and therefore would involve consent in a large group of patients for additional biopsies in the event of identification of malignancies, with only relatively small yields. This could potentially lead to further anxiety in patients, who have not yet been counselled about their potential findings in the same manner as that currently performed via the two-week wait route and would have to be assessed formally by REC. Therefore, to ensure that patients are adequately informed about their clinical condition without undue anxiety, research samples may need to be taken at a later date, when they have had sufficient time to assess whether they would like to participate in the study.

Hull & East Yorkshire Hospitals NHS Trust consists of two main sites, Castle Hill Hospital and Hull Royal Infirmary. Due to the location of the laboratory at Castle Hill Hospital, patients undergoing endoscopic biopsies were not recruited from the other site, as this would lead to delay in preparation of the biopsies for immediate placement within the microfluidic devices and thus affect the stringent protocols in place. However, in view of the ability to cryopreserve samples immediately upon collection for subsequent use without obvious alterations in morphology or behaviour, this would suggest that future samples could be collected from other centres, as well as both sites at Hull & East Yorkshire Hospitals NHS Trust.(376) Multiple advantages have been cited in the use of multi-centre-trials including: rapid recruitment, generalisability of results, potential inclusion of a wide range of population groups, use of contributions from multiple investigators with complementary expertise and the associated extensive quality control.(500,501) However, these studies do have several disadvantages, including the requirement of a co-ordinating centre to ensure standardisation and other issues generally relating to logistics, as well as the increased associated costs.

Although this was a pilot study, it may suffer from the use of low numbers of patients, in addition to the low number of replicates used for analysis. As briefly discussed statistical error, particularly type II errors are increased as a result of low numbers, thus missing potential alterations and accepting the null hypothesis. Greater patient numbers would consequently yield greater sample numbers and increase the power of the study to detect a practical difference when one actually occurs.

4.8.2 Representative tumour biopsy

Although efforts were made to take biopsies from viable areas of the tumour, there is always the possibility that areas of this tissue may be necrotic and therefore less likely to remain viable within the microfluidic device. This method of analysis also thus fails take into account tumour heterogeneity, where tumour cells can be highly diverse and sampling may not accurately reflect response of the whole tumour.(502–504) Therefore it is of particular importance that multiple tumour biopsies are studied and taken from a variety of areas throughout the tumour to be potentially representative.

4.8.3 Tumour functionality

Although successful demonstration of tissue viability has been determined based on morphology and LDH release in initial optimisation experiments, the functional status of the tissue after maintenance has not been evaluated. This has been previously successfully demonstrated using the microfluidic platform by Hattersley *et al* where the production of albumin and urea by the interrogated murine liver tissue was assessed.(378) P-glycoprotein transporters have been shown to be expressed in colorectal tissue and are responsible for cellular efflux to prevent substrate accumulation.(505–507) Kauffman *et al* assessed P-glycoprotein transport activity for given substrates to demonstrate functionality of human intestinal cell monolayers and with further adaptation of the technique, functionality of the tissue after microfluidic maintenance could also be evaluated.(508)

4.8.4 Radiation delivery and fractionation

Despite being unable to maintain tissue for the extensive duration reported by Carr *et al*, human rectal cancer tissue was maintained for a sufficient period of time to permit irradiation and evaluation of response through two distinct approaches: analysis of the tissue and also evaluation of the collected effluent after perfusion of the specimen.(381) Tissue was maintained for approximately 24 hours after placement within the microfluidics device, to allow initial acclimatisation prior to exposure to irradiation and then subsequently for a further 24 hours, to assess alterations secondary to radiation.

Although the microfluidic device itself is very compact, due to the relatively large spatial requirements of the microfluidic system used for irradiation within the Perspex prism and ensuring continuous perfusion described in section 2.7.4 and 2.9.2, only one sample could be transported to the clinical radiotherapy suite at one time without compromising the stringent protocols used. It was also important that only one device was in the suite at any one point, to prevent potential additional radiation exposure. However, future work would require irradiation of more than a single sample to ensure that findings were reproducible amongst individual patients and also to increase the power of the study and reduce the possibility of type II errors as discussed in section 4.8.1.

Ideally, the microfluidic devices would be in close proximity to the linear accelerator, therefore requiring minimal transportation and irradiation not be subject to clinical requirements to allow complete standardisation of the timing of radiation delivery. Use of a laboratory bench top irradiator would remedy these issues. Haff et al's review has shown that use of x-ray tube based delivery cabinets can act as an effective alternative to gamma irradiation, permitting laboratory-based irradiation.(509) However, the authors commented that dose uniformity varied dramatically with distance from the source and therefore significant radiation planning would be required prior to interrogation of tissue. An example of a commercial x-ray irradiator. the Faxitron has been successfully used in animal irradiation and therefore could be potentially utilised for future work.(510) The timing of radiation delivery was chosen based on the high levels of LDH seen on initial placement within the device and subsequent settling of release approximately 24 hours later as demonstrated in this study as well as in the work performed by Carr *et al.*(381) Due to this period of preservation, the effect of fractionated therapy was not assessed in this study. However, given that LDH is deemed to unlikely be successful as a biomarker of radiosensitivity, radiation delivery could be carried out immediately after placement within the device and permit a sufficient window of time in which fractionated doses of radiation could be delivered. This is a vital addition to future studies in the prediction of response to rectal cancer to LCCRT, where radiotherapy is delivered in repeated fractions and therefore may be more relevant.

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4.8.5 Concurrent chemotherapy

Chemotherapy has been delivered in conjunction with radiotherapy preoperatively to improve loco-regional control and systemic tumour control and has been attributed to radiation sensitisation.(116,511–513) The long term of role of 5-FU based chemotherapy in neo-adjuvant therapy for locally advanced rectal cancer has been demonstrated in the large EORTC 22921 randomised control trial (n = 1011) to be beneficial, by improving likelihood of ten year local recurrence (p = 0.0017).(514) However, this did not appear to translate with an improvement in ten-year disease-free or ten-year overall survival. The authors did not note a significant increase in side effects with the addition of chemotherapy. Hattersley *et al* successfully investigated the effect of chemotherapy including 5-FU and cisplatin on HNSCCs using this platform and therefore if fractionated radiotherapy with concurrent administration of chemotherapy were delivered to the interrogated tissue, it would emulate that delivered *in vivo*.(380,382)

4.8.6 Timing of effluent collection

The flow-rate through the microfluidic device and two-hourly effluent collections were based on the work of previous groups that have used the microfluidic device to ensure that tissue remained viable and that sufficient volumes of an appropriate concentration could be analysed for measurable compound alterations.

In this pilot study, effluent collections were performed at two-hourly intervals throughout the day and overnight for durations of up to 16 hours. Therefore, any activity potentially occurring in these overnight intervals would not be accounted for in the measurement of LDH and M30 ELISA. In addition, as discussed section 4.5, metabolomics analysis was only performed on these overnight collections due the limited quantity of effluent remaining after use for LDH analysis and M30 ELISA. Therefore this study is unable to account for changes outside these timeframes, however, with multiple simultaneous microfluidics devices, these investigations could be run in parallel. Collection of effluent on a two-hourly basis is potentially very labour intensive and it was for this reason that this was only performed during daytime hours. Refinement of the microfluidics system for future use, with addition of an automated fraction collector would ease this workload, allowing full evaluation of these time periods.

4.8.7 Future biomarkers

This study has mainly focussed on markers of necrosis and apoptosis and other mechanisms of radiation induced cell death have not been accounted for, such as mitotic catastrophe and senescence discussed in section 1.12.1. Vitale *et al* have reviewed potential markers of mitotic catastrophe and identified increased β -galactosidase activity, cell flattening and decreased telomerase function.(191) β -galactosidase activity has also been associated with senescence, however, very few authors have previously used it to assess response to radiation.(515–518) Coates *et al* noted induction of β -galactosidase after irradiation of transgenic mice.(515) Chen's study of xenografts of breast cancer cell lines used β -galactosidase staining, with significantly greater staining in irradiated tumours when compared to the controls (p < 0.05).(517) It does not appear that this method has been used in the investigation of rectal cancer, but it may be worth determining if mitotic catastrophe and senescence play a significant role in response to radiotherapy.

Given the success of this platform in the interrogation of rectal cancer tissue, future work should also increase the panel of biomarkers assessed and focus on the markers discussed in section 1.13 found to be suggestive of response, with EGFR, bax and survivin in particular. Further metabolomics work should also focus on the three compounds identified by Kim's group.(493) However, it is imperative that a greater number of tissue samples are available to perform these considerable tasks.

4.8 Conclusions

This work has demonstrated that the microfluidic device can be used to reliably maintain both *ex vivo* healthy murine colorectal and human rectal cancer tissue for a sufficient period of time, to permit interrogation with radiation. Findings demonstrate that apoptosis and morphological changes are induced by irradiation, but further work is required to expand the panel of biomarkers assessed and to correlate findings with clinical outcome. However, important progress has been made to allow use of this platform as a predictive tool of response to neo-adjuvant therapy to truly deliver personalised therapy.

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Appendix 1)

A comparison of Mandard and Dworak tumour regression grading(135,136)

Mandard		Dworak	
Grade	Complete response with	Grade	No regression
1	absence of residual cancer	0	
	and fibrosis extending through		
	the wall		
Grade	Presence of residual tumour	Grade	Dominant tumour mass with
2	cells scattered through the	1	obvious fibrosis and/or
	fibrosis		vasculopathy
Grade	Increase in the number of	Grade	Dominantly fibrotic changes with
3	residual cancer cells, with	2	few tumour cells or groups (easy to
	fibrosis predominant		find)
Grade	Residual cancer outgrowing	Grade	Very few (difficult to find
4	fibrosis	3	microscopically) tumour cells in
			fibrotic tissue with or without
			mucous substance
Grade	Absence of regressive changes	Grade	No tumour cells, only fibrotic mass
5		4	(total regression or response)

Appendix 2)

Research protocol

1.1 Long title

An observational pilot study to assess the potential of a microfluidic tissue culture model to predict rectal cancer response to neo-adjuvant therapy.

1.2 Short title

Microfluidic analysis of rectal cancer response to neo-adjuvant therapy.

1.3 Protocol number

ASU-IAH-2011.1

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2.1 Abbreviations

Magnetic Resonance Imaging	MRI
Multidisciplinary team meeting	MDT
Computerised axial tomography	СТ
Short course radiotherapy	SCRT
Long course chemoradiotherapy	LCCRT
T stage	tumour
N stage	nodal
M stage	metastases

3.1 Background

Rectal cancer is diagnosed in 14000 people a year in the UK (Cancer Research UK 2004). The most effective treatment consists of total mesorectal resection, an operation in which the rectal cancer is removed along with the mesenteric fat that surrounds it. This is a highly invasive procedure associated with significant morbidity and mortality (Tekkis et al., 2003). Despite improvements in surgical technique, the development of local tumour recurrence and systemic metastases is common (Douillard et al., 2000). The administration of chemotherapy and pelvic radiotherapy can improve outcomes in selected patients (Sebag-Montefiore 2006). At present the administration of these treatments is guided by the radiological and histological determination of tumour stage and grade. However, attempts to predict tumour behaviour based on stage and grade are less than accurate, resulting in both the under treatment and overtreatment of a proportion of patients. Analysis of tumours following surgical resection after the administration of neoadjuvant therapies indicates that some tumours are very resistant to chemoradiotherapy, while others demonstrate a complete response

(Horisberger *et al.*, 2008). If surgeons and oncologists had the ability to predict which tumours would and would not respond to such neo-adjuvant treatments they could administer these interventions in a much more selective and efficient manner. This would avoid the potential for treatment related side effects and toxicity in patients who would not clinically benefit from this form of treatment. It is also possible that the ability to predict total response could influence the nature of surgical treatments by enabling a surgeon to perform a local or less radical resection of the rectal cancer.

As a surgical unit that specialises in the treatment of rectal cancer we are in the privileged position to directly observe the effects of neo-adjuvant treatments on our patient's tumours. Once a patient has been diagnosed with a rectal cancer they are fully assessed with tissue biopsy and radiological imaging. Each patient has their treatment determined by a panel of oncologists, radiologists and surgeons. Many patients will be recommended for long course pre-operative chemoradiotherapy. Once treatment is completed the patients cancer will be removed by surgical resection and the resection specimen analysed by a pathologist. This enables the pathologists to determine how well the cancer has responded to the treatment. This standard system of treatment provides the perfect model for the development of methods that can predict treatment response.

This study aims to develop such a model using new tissue culture techniques developed in the University of Hull by Professor Greenman and his team. Small pieces of a rectal cancer can now be taken prior to the start of treatment for growth or culture in a small glass chamber. Samples of a rectal cancer can be kept alive in this way for 7 to 10 days. These samples can be used as a model of a patient's individual rectal cancer. By exposing these samples to radiation we hope to be able to develop a method that will allow us to predict exactly how that cancer will respond to radiation therapy in the individual patient. Based on the current volume of rectal cancer resection in our unit we would predict that over 50 patients will receive long course chemoradiotherapy in the next 24 months.

3.2 Aims and objectives

This study will aim to develop a tissue culture model of rectal cancer that can be used to predict how an individual cancer will respond to chemoradiotherapy. In the long term our objective is to use this model to plan the use of chemotherapy, radiotherapy and surgery more effectively in individual patients.

3.3 Study design

This is an observational study of a cohort of patients with rectal cancer receiving neo-adjuvant therapy prior to surgery. The analysis of tissue samples derived from this cohort will be undertaken using molecular biology techniques in a designated laboratory.

This trial has been designed to have an almost negligible impact on the current routine therapy applied to patients with rectal cancer.

Rectal cancer is almost always diagnosed by endoscopic (telescopic) examination of the colon and rectum. This enables direct tissue biopsy of the cancer using endoscopic forceps. Biopsies are taken to confirm the cancerous nature of rectal lesions prior to the initiation of intensive and potentially harmful treatments.

Following diagnosis all patients are radiologically staged with a pelvic and abdominal magnetic resonance (MRI) scan and a computed tomogram (CT) of the thorax. These investigations provide all the information required to formulate a treatment plan. Each patient's treatment is determined at a multi-disciplinary surgical oncology meeting (MDT). The majority of patients with rectal cancer will be recommended for some form of neo-adjuvant therapy (treatment with chemotherapy and/or radiotherapy before surgery). This involves either a 5-day course of radiotherapy over a 5-week period (LCCRT). These treatments are designed to kill cancer cells and are known to improve the effectiveness of surgery in removing cancers from the rectum and pelvis. The LCCRT is specifically used for larger tumours and usually manages to shrink or down stage the cancer prior to an operation. Once a patient has been

diagnosed with a rectal cancer they will be considered for entry into this research. Patients will be identified following presentation to an outpatient clinic. Any patient who is referred from these clinics for full colonoscopic examination due to a suspected rectal cancer will be asked if they wish to participate. At this point they will receive a trial information pack, which they can read in the interim between presentation and attendance for colonoscopy (usually about a week). On the day of colonoscopy each patient will be formally approached by a member of the research team and consented to participate if they desire. During colonoscopy the rectal lesion will be evaluated. Samples will be obtained by brushing and by endoscopic forceps biopsy. If a patient has agreed to take part in the research we will take this opportunity to perform a further forceps biopsy of the rectal cancer. This will involve taking around 10 biopsies as opposed to the usual 5. This is done using an endoscopic forceps via the telescope to take several bites about 2mm in size from the cancer. As the lining of the bowel has no pain receptors this biopsy will not cause any pain. The patient may experience the passage of small amounts of blood after the procedure but this is almost always self-limiting and very rarely requires treatment. These biopsies will be performed by either a consultant colorectal surgeon or a colorectal research fellow. All colonoscopies will be supervised by a Joint Association of Gastroenterologists accredited colonoscopist. The tissue obtained at biopsy will then be transported to the laboratory and processed for tissue culture in a microfluidic chamber. This extra biopsy is the only additional intervention that each patient will receive. Other than this each patient's treatment will be exactly the same as a patient not involved in the research. However, data pertaining to subjects will continue to be collected for 5 years.

If the patient's histological biopsy taken at colonoscopy confirms the lesion to be cancer, the patient may undergo surgery after their neadjuvant treatment (either LCCRT or SCRT).

During surgery the patient will undergo a routine examination under anaesthetic and the tumour will be resected. We would also like to recruit patients that have not undergone neoadjuvant therapy to take biopsies at this stage.

As this cancerous tissue is being removed, there is no additional risk to the patient of the biopsies at this time. The biopsies taken will not affect the histological staging of the cancer specimen.

Each subject's data set will be anonymised but will include the patient's demographic characteristics (i.e. age, sex etc.). The details of each patient's adjuvant therapies and their subsequent operations will also be recorded. After surgical resection of a cancer it is standard practice for a pathologist to analyse the resected tissue and record the stage of the cancer. This also allows the pathologist to grade how well a cancer has responded to neo-adjuvant therapy on a scale of 1 to 5 (5 being no response at all to 1 being a complete resolution of the cancer). This regression will be recorded. General outcomes of treatment will also be recorded including surgical complications, disease free survival and overall survival. This data will be collected for five years after surgical resection.

Five biopsy specimens should provide enough tissue to establish two parallel microfluidic cultures. These tissue cultures will be grown in the lab for a maximum of 10 days. During that time the cultures will be subjected to external beam radiotherapy. Much of the initial work will be aimed at establishing the correct dosage of radiation to induce detectable changes of radio-resistance or sensitivity. This dose will have to be high enough to damage the cells but not to kill them. The second phase of the project will involve measuring a variety of parameters in an attempt to identify quantifiable changes that reflect radio-resistance or sensitivity in the parent cancer treated by LCCRT. The microfluidic system is ideal in that it allows an almost minute-by-minute analysis of the tissue cultures. Morphological changes in the cultures can be identified by light microscopy. Changes in protein and metabolite excretion can be assessed by analysis of the fluid that the cells are grown in. Changes in DNA expression can be tracked with real time polymerase chain reaction. Assays to determine cell death rates, cell DNA damage and changes in cell membrane permeability can also be applied.

3.4 Endpoints

Quantifiable molecular changes in tissue cultures derived from rectal cancers following radiation treatment

The response of rectal cancers to LCCRT in patients as determined by histopathological grading of Mandard regression.

3.5 Subject selection and recruitment

Patients will be identified and recruited from the outpatient clinics of the Castle Hill Hospital Department of Colorectal Surgery. On average the department treats 10 rectal cancers per month, which will translate into over 100 cases per year. It is predicted that at least 60% of patients will agree to participate in this study. Given the negligible impact on patient experience of the study design it is hoped that this proportion of recruitment will be exceeded.

Once a patient has been identified for further investigation on the suspicion of rectal cancer they will be asked if they are interested in entering the trial. If they agree in principal then they will be given written information on the trial protocol that they can read at their own convenience (**Appendix 4, Patient information sheet**).

As part of their investigation these patients will all have a flexible endoscopy to allow tissue biopsies to be taken in order to confirm their diagnosis. This investigation usually occurs within a week of identification in the outpatient clinic. When they attend for endoscopy they will be approached by a member of the research team and entry into the study will be discussed again. If the patient agrees to study participation they will be formally consented (**Appendix 5, Consent form**).

If a patient is diagnosed to have rectal cancer, but will not be undergoing neo-adjuvant therapy, they will be approached by a member of the research and if they agree in principal, then they will be given written information on the trial protocol that they can read at their own convenience (**Appendix 4, Patient information sheet**). If the patient agrees to study participation they will be formally consented

(Appendix 5, consent form).

As with all clinical interactions, patients who lack sufficient English language skills to understand the implications of consent will be provided with a qualified and independent interpreter. Patients who are judged to lack sufficient mental capacity to consent will be excluded from the study. Patients will not be offered any remuneration for trial participation. Once enrolled in the study each patient will be allocated an individual study number and study file. This file will document the patient's demographic details and aspects of their diagnosis and subsequent treatment. Individual trial numbers will be used to anonymise each patient's trial file so that patients can only be individually identified from their documentation by access to the list of trial numbers by patient name. These documents and the list will be kept separately and in a secure office accessible only to the research team.

3.6 Inclusion criteria

Any patient presenting to Castle Hill Hospital with a presumed diagnosis of rectal cancer that requires full colonoscopy, biopsy, pelvic MRI and thoracic CT.

3.7 Exclusion criteria

Pregnant women. Patients unable to consent to participation. Presence of a significant coagulopathy. Previous pelvic radiotherapy.

3.8 Randomisation

This study design does not require randomisation.

3.9 Blinding

This study design does not require participants or experimenters to be blinded.

3.10 Compliance

If patients opt not to have resectional surgery or neo-adjuvant treatments, then they will be given the option of having their collected samples destroyed and they will not be included in any further analysis. If patients cannot be contacted after such non-compliance then their samples will be destroyed and they will not be included in any further analysis. If patients fail to comply with the standard surveillance and follow up protocols offered to rectal cancer patients, then they will be censored from any further long-term analysis.

3.11 Subject withdrawal

If a patient wishes to withdraw from the study they will be given the option of having their existing samples destroyed and they will not be included in any further analysis. If patients become unable to withdraw through death or deterioration in mental capacity, their already donated samples will still be analysed, but the continued recording of clinical data will be stopped.

3.12 Data collection

Patient demographic data will be collected at the time of trial consent and recorded in the patient's trial file. This data will include:

- Date of presentation
- Age at presentation
- Gender
- Ethnic origin
- Smoking history
- Family history of bowel cancer
- Previous surgical history
- Previous history of bowel cancer
- Significant co-morbidities

The clinical details of a patient's rectal cancer will be derived from the patient case notes and the electronic reports of relevant imaging and pathological analysis. This data will include:

- Level of cancer from the dentate line
- MRI T and N stage
- MRI and CT M stage
- A copy of the pathological report obtained by histological analysis of the subject's resection specimen following surgery.

The clinical details of a patient's treatment will be obtained from the patient's notes and a copy of the MDT discussion notes. This will include:

- Neo-adjuvant treatment administered
- Date of neo-adjuvant treatment
- Symptoms of treatment toxicity
- Surgical procedure
- Date of surgery
- Post-operative complications within 30 days of surgery

The details of clinical progress of the patient for 5 years after treatment will be recorded from patient notes. This will include:

- Evidence of local tumour recurrence
- Evidence of distant metastasis
- Death and cause of death

(Appendix 2, data collection forms)

3.13 Tissue collection

Tissue samples will be collected as described in the study methods. This process will abide with the rules and guidelines set out by the Human Tissue Act. Each sample will be labelled using each patient's individualised trial number. Following 10 days of culture the remaining viable cells may be fixed in formalin or methanol for subsequent microscopic analysis. Any residual sample may be kept for a period of 5 years and will then be destroyed.

4.1 Ethical considerations

The ethical implications of this study are those related to the collection and storage of Human derived tissues. This process will abide with the rules and guidelines set out by the Human Tissue Act. All patients will be informed of the intention to obtain, store and analyse their tissue samples during the consent process. This will include the intention to keep these samples for up to five years, and arrangements in place to dispose of these samples at the termination of the study.

4.2 Ethical approval

The study will be performed subject to Research Ethics Committee favourable opinion and HEY Trust R & D approval.

4.3 Research Governance

This study, where applicable, will be conducted in accordance with The Medicines for Human Use (Clinical Trials) Regulations 2004 and Amendment Regulations 2006 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care.

4.4 Data recording and handling

Mr Iain Andrew Hunter will be responsible for all data collection, recording, storage and data quality.

IT Services Department has a backup procedure approved by auditors for disaster recovery. Servers are backed up to tape media each night. The tapes run on a 4-week cycle. Files stay on the server unless deleted by accident or deliberately. Anything deleted more than 4 weeks previously is therefore lost. Additional 'archive' backups are taken for archived data, so data should not be lost from this type of system e.g. FileVision, which stores Medical Records. Tapes are stored in a fireproof safe. Study documents (paper and electronic) will be retained in a secure (kept locked when not in use) location during and after the trial has finished. All essential documents including source documents will be retained for a minimum period of 5 years after study completion (last patient, last visit). Data will be collected and retained in accordance with the Data Protection Act 1998.

4.5 Access to Source Data

The Investigator will permit monitoring, audits, REC and MHRA review (as applicable) and provide direct access to source data and documents.

5.0 Indemnity

This is an NHS-sponsored research study. If there is negligent harm during the trial when the NHS body owes a duty of care to the person harmed, NHS indemnity covers NHS staff and medical academic staff with honorary contracts only when the trial has been approved by the Trust R & D department. NHS indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Where the Chief/Principal investigator is employed by the University of Hull, the University has an insurance policy that includes cover for no-fault compensation in respect of accidental injury to a research subject.

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Tekkis PP. Poloniecki JD. Thompson MR. Stamatakis JD. (2003) Operative mortality in colorectal cancer: prospective national study. BMJ. 327(7425):1196-201.

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Appendix 3)

Data collection form

Trial number	M/F	
Date of presentation	Age at	
	presentation	
Ethnic origin		
Smoking history		
Family history of bowel		
cancer		
Previous surgical history		
Previous history of bowel		
cancer		
Significant co-morbidities		
Level of cancer from		
dentate line		
MRI T and N stage	MRI/CT M stage	
Histopathology/Response		
to neo-adjuvant therapy		
(1-5)		
1 - Complete resolution		
2 – No response		
MDT		
Neo-adjuvant treatment	Date of neo-	
	adjuvant	
	treatment	
Symptoms of treatment		
toxicity		
Surgical procedure	Date of surgery	

Post-op complications	
(30 days)	
Trial number	
Evidence of local tumour	
recurrence	
Evidence of distant	
metastasis	
Death and cause of death	

Appendix 4)

Patient information sheet

An observational pilot study to assess the potential of a microfluidic tissue culture model to predict rectal cancer response to neo-adjuvant therapy.

You are being invited to consider participating in a research study. We would like you to read the following information and then carefully consider whether or not you would like to take part. You do not have to make a decision right now, but we will ask you about taking part again when you attend for more tests in the next few days. We will be more than happy to answer any questions you may have at that time and if you want to take part we will ask you to sign a consent form to confirm your wish to participate.

(Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Part 1

What is the purpose of this trial and why have I been chosen to take part?

During your investigations today we have detected an abnormal growth in your lower bowel. The Doctor or Nurse who has seen you today will have explained to you that there is a possibility that this growth is a type of bowel cancer. This study is designed to develop a new test to improve treatment effectiveness for patients in the future. This trial will also form part of a research degree and thesis for a student completing an MD at The University of Hull.

Do I have to take part?

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Are there any reasons why I should not take part?

You will not be eligible to take part if you are pregnant, if you have a blood clotting disorder, you are less than 18 years old or if you have had previous pelvic radiotherapy.

What will I have to do if I agree to take part?

This study has been designed to have an almost negligible impact on the way you would normally be treated. In order to establish the nature of the growth in your bowel we recommend that you have a number of further tests. These will usually include a magnetic resonance scan (MRI), a CAT scan and a repeat telescope test called a colonoscopy. The colonoscopy will be performed to look at your entire colon and to allow us to take some biopsies from the growth in your bowel. All these tests will be

recommended to you regardless of whether or not you decide to participate in this research study.

If you decide that you would like to take part in this study we will ask you to consent to have more biopsies taken from your bowel than would normally be the case. During routine treatment we would normally take 5 small pieces (about 2mm in size, smaller than the size of a match head) from your bowel growth using a small pair of forceps that are passed down the colonoscope. If you agree to participate in the study we will increase this number to 10 pieces in total.

If you do require treatment for your bowel we will collect information about the type of treatment that you have and how well you respond to that treatment. Depending on how you respond to treatment we may decide to analyse the tissue from your bowel. The purpose of this analysis is to determine the different proteins that are present in your biopsy specimen. By performing this analysis we hope to identify proteins that can help predict how different patients will respond to different types of treatment.

Will my treatment be affected if I decide that I do not want to take part?

No. The investigations and treatments that we will recommend and offered to you will be identical regardless of your decision. The only difference will be that we will take 10 biopsies rather than 5 at the time of your colonoscopy and/or at the time of surgery.

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Are there any additional risks to my health if I do take part?

Taking these biopsies form your lower bowel is considered to be very safe and the additional risk to you above that of a routine colonoscopy and biopsy is negligible. Taking 5 more biopsies may increase the duration of your colonoscopy but this increase should be less than 10 minutes. As the lining of your bowel does not have the same type of pain receptor nerves as your skin you should not experience any pain from the biopsies. You may experience some bleeding from the anus after this procedure but this is almost always short lived and rarely requires any treatment. You are likely to experience this bleeding regardless of whether or not you are taking part in the study, as you will require at least 5 biopsies as a part of your normal treatment.

Should you require surgery, there is no additional risk associated with biopsies taken at this time, as these biopsies will be taken from the cancer specimen being removed.

Will there be any direct benefit to my care if I take part?

No. Your participation in the study will have no beneficial or detrimental impact on your care. However, it is hoped that the findings of this research will benefit patients who present with similar conditions in the future.

Will participation in the study require me to do anything else once I have had my biopsies taken?

Other than gifting us with these extra biopsy specimens your treatment throughout will be identical to that of a patient who is not taking part in the study. However, we will also ask you to consent to allow us to collect some extra information about you and your future treatment over the next 10 years. This will not require any additional work on your part as this information will be collected from your NHS paper and electronic records by a member of the research team.

Part 2

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the HEY NHS Trust Research and Development Department and has also been reviewed and approved by South Yorkshire Research Ethics Committee.

Will my taking part in the trial be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

Will my family Doctor know that I am taking part?

No.

What will happen to the information that you collect about me?

If you agree to take part in this trial you will also be asked to grant the research team direct access to your medical notes. Any data collected from you will be stored in a data file that will be kept in a secure office. Data analysis will require a computer. However, any data stored on a computer will not include any personal information that can be used to identify you in any way. Data will be stored for ten years in a safe archive. After ten years the data will be destroyed. The results of this research may be published in the form of an article in a peer reviewed medical research journal. Such an article will not contain any personnel data that could be used to identify you. If you want to know more about the results of the trial when it is finished you can request a written summary that will explain all the main findings of the research.

What kind of information will you be recording and storing?

The information that we will record will include:-

Your age today

Your gender

The results of any MRI or CAT scans that you have in relation to your treatment

Details of any treatment that you receive for the growth in your bowel, including any operations that you have

Details of any microscopic analysis of your biopsy specimens or specimens removed at operation in the future Details of how you respond to any treatments that you may require in relation to your bowel condition

What will happen to the tissue that I have donated to the study?

All tissue samples will be collected and stored in accordance with the regulations of The Human Tissue Act. Once the biopsies are taken they will be collected by a member of the research team and stored in a deep freezer. This freezer is situated in a secure laboratory and is only accessible to members of the research team.

All your donated tissue samples will be stored for 5 years. After 5 years all the samples will be destroyed unless the research team has gained ethical approval through the National Research Ethics Service to use them in a new study.

Will I receive any remuneration if I take part?

No. Taking part will not incur any expense to you and will be a totally altruistic undertaking.

What will happen if I decide that I no longer wish to take part in the study?

You can withdraw your consent to have biopsies taken or information collected from your records at any time. If you withdraw consent after your biopsies have been taken and analysed it will not be possible to exclude them from the final analysis. However, any residual tissue that has not been processed will be destroyed and no further information will be collected about you for the purpose of the study. If you do withdraw your consent this will have no impact on your continued treatment.

What can I do if I want to contact the research team in the future? If you need to talk to the research team they can be contacted by calling 07427625234

Or by writing to Radiobiology Study 2 Academic Unit of Surgery Castle Hill Hospital Cottingham HU16 5JQ

Or by e-mailing

radiobiology2@hunter.prestel.co.uk

What will happen if I come to any harm as a result of this study?

It is very unlikely that you will be put at additional risk if you take part in this trial. If you do suffer any unexpected adverse events during this research you are not immediately entitled to compensation. In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against The Hull and East Yorkshire Teaching Hospitals Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate). Should you suffer any unexpected adverse events during the trial period your participation in the trial will be stopped.

Appendix 5)

Consent form

Centre Number:

Study Number:

Patient Identification Number for this trial:

CONSENT FORM

Title of Project:

An observational pilot study to assess the potential of a microfluidic tissue culture model to predict rectal cancer response to neo-adjuvant therapy.

Name of Researcher:

Please initial box

1. I confirm that I have read and understand the information sheet dated 6 August 2012 (version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I understand that the tissue that I donate during this study will be handled in accordance with the Human Tissue Act. It will be stored for 5 years at which point it will be destroyed unless approval is sought and gained through the National Research Ethics Service for use in a new or related study.

5. I consent for the tissue that I donate during this study to be used in future new or related studies if approval is gained through the National Research Ethics Service.

6. I agree to take part in the above study.

Name of Patient	Data	Signature	
	Date	Signature	
Name of Person	Date	Signature taking consent	
When completed, 1 fo	r patient; 1 for r	esearcher site file; 1 (original) to be kept in medical not	ies

I would like to receive a summary of the study findings when the project has been completed. This can be sent to the address or e mail address below.
Appendix 6)

Supplying companies

Company	Address	Contact details
AbD Serotec [®]	Endeavour House	Phone: +44 (0)1865 852700
	Langford Lane	Fax: +44 (0)1865 852739
	Kidlington	Email: abd_sales_uk@bio-
	Oxford	rad.com
	OX5 1GE	Website: www.abdserotec.com
	UK	
Autodesk Inc.	One Discovery	Phone: +44 (0)1252 456600
	Place	Fax: +44 (0)1252 456601
	Columbus Drive	Website: www.autodesk.co.uk
	Farnborough	
	Hampshire	
	GU14 ONZ	
	UK	
B & K Universal Ltd.	Grimston	Phone: +44 (0)1964 527555
	Aldbrough	Fax: +44 (0)1964 527006
	Hull	Website: www.bku.com
	HU11 4QE	
	UK	
B & Q	B&Q House	Phone: 03330143098
	Chestnut Avenue	Website: www.diy.com
	Chandler's Ford	
	Eastleigh	
	SO53 3LE	
	UK	
BD (Becton, Dickinson and	The Danby	Phone: +44 (0)1865 781666
Company)	Building	Fax: +44 (0)1865 781627
	Edmund Halley	E-mail:
	Road	Orders.uk@europe.bd.com
	Oxford Science	Website: www.bd.com/uk
	Park	
	Oxford	
	OX4 4DQ	
	UK	

Biosera Ltd. c/o	International Ltd	Phone: +44 (0) 1273 814888
Labtech	2 Birch House	Fax: +44 (0) 1825 766492
	Brambleside	Website: www.biosera.com
	Bellbrook Industrial	(www.labtech.com)
	Estate	Email: sales@labtech.com
	Uckfield	
	East Sussex	
	TN22 1QQ	
	UK	
Boxer Laboratory	Unit 1 Hitchs Yard	Phone: +44 (0)1920 468727
Equipment Ltd.	Church Street	Fax: +44 (0)1920 468644
	Ware	
	Hertfordshire	
	SG12 9ES	
	UK	
Bruker (UK) Ltd.	Banner Lane	Phone: 024 7685 5200
	Coventry	Website: www.bruker.com
	CV4 9GH	
	UK	
Covatutto	c/o Hatch-It	Tel: +44 (0)1635 230 238
	Incubators	Fax: +44 (0)1635 349 20
	Palady Spring, Old	Website:
	Andover Road	www.hatchitincubators.com
	Newbury	
	RG20 OLS	
	UK	
Dako UK Ltd.	Cambridge House	Tel. 5 44 (0)1353 669911 5
	St Thomas Place	Fax 5 44 (0)1353 668989 5
	Ely	E-mail: info.uk@dako.com
	Cambridgeshire	Website: www.dako.com
	CB7 4EX	
	UK	
Dow Corning	Dow Corning Europe	Phone: +32 64 888 000
Corporation	SA	Fax: +32 64 888 401
	Parc Industriel - Zone	Website: www.dowcorning.com
	С	
	Rue Jules Bordet	
	7180 Seneffe	
	Belgium	

ESCO GB Ltd.	Unit 21	Phone: +44 (0)1226 361529
	Shortwood	Fax: +44 (0)1226 741709
	Business Park	Email: egb.info@escoglobal.com
	Shortwood Close	Website: ww.escolifesciences.eu
	Hoyland	
	Barnsley	
	S74 9LH	
	UK	
Elekta Ltd.	Linac House	Phone: +44 (0)1293 544422
	Fleming Way	Fax: +44 (0)1293 654321
	Crawley	Website: www.elekta.com
	RH10 9RR	
	UK	
GraphPad	7825 Fay Avenue	Phone: 858-454-5577
Software Inc.	Suite 230	Fax: 858-454-4150
	La Jolla	Email: sales@graphpad.com
	California 92037	Website: www.graphpad.com
	USA	
Harvard	P.O. Box 126	Phone: +44 (0)1732 864001
Apparatus Ltd.	Edenbridge	Fax: +44 (0)1732 863356
	Kent	E-Mail: sales@harvardapparatus.co.uk
	TN8 6WF	Website: www.harvardapparatus.co.uk
	UK	
IDEX Health &	Futtererstrasse 16	Phone: +49 (0) 1801 808 800 L
Science (Idex	97877 Wertheim-	Fax: +49 9377 1388 sep
	Mondfeld	CustomerService.hseurope@idexcorp.com
	Germany sep	Website: www.upchurch.com
Leica	Larch House	Phone: 0800 298 2344
Microsystems	Woodlands	Fax: +44 (0)1908 577 640
UK Ltd.	Business Park	Website: www.leica-microsystems.com
	Breckland	
	Linford Wood	
	Milton Keynes	
	MK14 6FG	
16 11	UK	
Media	Beech House	Phone: +44 (0)1628 47/025
<i>Cybernetics Inc.</i>	27 Little Marlow	Fax: +44 (0)1628 891764
	Road	E-mail jhainsworth@mediacy.com
	Marlow	Website: www.mediacy.com
	Buckinghamshire	
	SL7 1HA	
	UK	

Microsoft	Microsoft	Telephone: 0844 800 2400
Corporation	Campus	Website: www.microsoft.com/en-
	Thames Valley	gb/default.aspx
	Park	
	Reading	
	RG6 1WG	
	UK	
MZmine		Website:
		mzmine.sourceforge.net/index.shtml
National	Unit 4 Fleet	Phone: 44 (0) 1482 646020
Diagnostics AGTC	Business Park	Email: office@agtcbioproducts.com
Bioproducts t/a	Itlings Lane	Website:
National Diagnostics	Hessle	http://www.agtcbioproducts.com
UK	Hull	
	East Riding of	
	Yorkshire	
	HU13 9LX	
	UK	
National Institutes	9000 Rockville	Website: http://imagej.nih.gov/ij/
of Health	Pike	
	Bethesda	
	Maryland 20892	
	USA	
Nikon UK Ltd.	380 Richmond	Tel: +44 (0)208-247-1717
	Road	Fax: +44 (0)208-541-4584
	Kingston Upon	Email: discover@nikon.co.uk
	Thames	Website:
	Surrey	www.nikoninstruments.com/en_GB
	KT2 5PR	
	UK	
Oxoid	Wade Road	Tel: +44 (0)1256 841144
	Basingstoke	Fax: +44 (0)1256 814626
	Hampshire	E-mail:
	RG24 8PW	<u>seoxoid.orders@thermofisher.com</u>
	UK	
PAA Laboratories	Healthcare Life	
c/o GE Distributed	Sciences	
	Amersham Place	
Sigma-Aldrich	Little Chairont	
Company Ltd.	BUCKINGNAMSNIFE	
(See below)	HP/9NA	
PEVIVA		Phone: +46 8 122 053 00
	Loistroms alle 5A	Fax: +46 8 / 30 16 10
		E-Mail: order@peviva.net
	66 Sundbyberg	

	Sweden	
Philips Healthcare	Philips Centre	Phone: +44 (0)1483 792206
	Guildford Park	Fax: +44 (0)1483 298842
	Guildford	
	Surrey	
	GU2 8XH	
	UK	
Riello UPS Ltd	Clywedog Road	Phone: +44 (0)1978 729 297
	North - Unit 50	Fax: +44 (0)1978 729 290
	Wrexham	
	Industrial Estate	
	Wrexham	
	LL13 9XN	
	UK	
Roche Diagnostics	Charles Avenue	Phone: +44 (0)1444 256000
Limited	Burgess Hill	
	West Sussex	
	RH15 9RY	
	UK	
Sakura Finetek UK	1 Thatcham	Phone: +44 (0)845 0701638
Ltd	Business Village	Fax: +44 (0)845 0701639
	Colthrop Way	Web: www.sakura.eu
	Thatcham	
	RG19 4LW	
	UK	
Sartorius UK Ltd.	Longmead	Phone: +44 (0)1372 737159
	Business Centre	Fax: +44 (0)1372 729927
	Blenheim Road	E-mail:
	Epsom	uk.customerservice@sartorius.com
	Surrey	Web: http://www.sartorius.co.uk
	KT19 9QQ	
	UK	
Sigma Aldrich	The Old Brickyard	Phone: 0800 717181
Company Ltd.	New Road	Fax: 0800 378785
	Gillingham	Email: ukorders@sial.com
	Dorset	Web: www.sigmaaldrich.com/united-
	SP8 4XT	kingdom.html
	UK	Pl 44 (0)44 4 00 4 4004
Swann-Morton Ltd.	Owlerton Green	Phone: +44 (0)114 234 4231
	Sheffield	Fax: +44 (0)114 231 4966
	S6 2BJ	Email: uksales@swan-morton.com
	UK	Website: www.swann-morton.com

Thermo Fisher	c/o Oxoid	Tel: +44 (0)1256 841144
Scientific	Limited	Fax: +44 (0)1256 814626
	Wade Road	E-mail: 🛵 xoid.orders@thermofisher.com
	Basingstoke	Website: www.oxoid.com/UK/blue
	Hampshire	
	RG24 8PW	
	UK	
Vector	3 Accent Park	Phone: +44 (0)1733 237999
Laboratories	Bakewell	Fax: +44 (0)1733 237119
Inc.	Road	E-Mail: vector@vectorlabs.co.uk
	Orton	Website:
	Southgate	https://www.vectorlabs.com/uk/default.aspx
	Peterborough	
	PE2 6XS	
	UK	
VWR BDH	Hunter	Phone: +44 (0)1455 558600
Prolabo (VWR	Boulevard	Fax: +44 (0)1455 558586
International)	Magna Park	Email: info@uk.vwr.com
	Lutterworth	Website: www.vwr.com
	Leicestershire	
	LE17 4XN	
	UK	

Appendix 7)

Tables demonstrating change in levels of caspase cleaved cytokeratin between effluent collected pre irradiation and post irradiation and at the same time points in the control group in three patient groups classified according to the concentration of the patient samples prior to the irradiated sample receiving radiation: A) Low (<100 Units/l/mg) (p = 0.0613), B) Medium (100-500 Units/l/mg) (p = 0.258) and C) High (>500Units/l/mg) (p= 0.390)

A) Low concentration group							
	Control t	Control tissue					
	concentr	ation		Irradiated ti	Irradiated tissue concentration		
	(Units/l/	'mg)		(Units/l/mg)	(Units/l/mg)		
Patient				Pre	Post		
sample	Pre	Post	Change	irradiation	irradiation	Change	
1A	16.2	17.9	1.72	20.2	20.7	0.570	
1B	17.0	16.2	-0.862	19.6	20.2	0.570	
7A	53.7	61.1	7.35	46.0	119	72.7	
7B	42.4	68.4	26.0	47.4	124	76.9	
8A	32.8	19.9	-12.9	50.8	32.4	-18.4	
8B	36.3	26.7	-9.64	37.5	29.1	-8.36	
11A	51.1	48.7	-2.45	21.5	197	176	
11B	51.7	47.5	-4.28	24.7	215	191	
Mean	37.657	38.3	0.628	33.5	94.8	61.3	
Standard							
deviation	15.0	20.7	12.0	13.4	80.8	83.5	

(Numbers to 3 significant figures or 1 decimal point)

B) Medium concentration group						
	Control tissue concentration (Units/l/mg)		Irradiated tissue concentration (Units/l/mg)		tion	
Patient				Pre	Post	
sample	Pre	Post	Change	irradiation	irradiation	Change
3A	160.0	394.3	234.3	29.0	63.4	34.4
3B	122.0	308.2	186.2	11.5	21.2	9.67
4A	14.5	17.4	2.95	106.1	233.1	127.1
4B	14.9	17.8	2.95	122.7	267.8	145.1
5A	225.0	313.0	88.0	28.9	47.2	18.2
5B	242.4	343.2	100.8	33.4	61.8	28.4
6A	28.3	27.6	-0.700	191.3	110.0	-81.8
6B	24.8	26.9	2.10	213.7	137.0	-76.8
Mean	104.0	181.1	77.1	92.1	117.6	25.5
Standard						
deviation	96.5	171.6	92.5	79.0	90.0	81.9

C) High concentration group						
	Control tissue concentration			Irradiated tissue concentration		
		(Un	its/l/mg)	(Units/l/mg)		
Patient				Pre	Post	
sample	Pre	Post	Change	irradiation	irradiation	Change
2A	74.1	37.9	-36.3	492.4	1053.4	561.0
2B	79.5	26.6	-52.9	511.9	1173.5	661.6
9A	1112.3	577.8	-534.9	1236.7	363.1	-873.2
9B	1152.3	518.3	-634.1	1204.9	350.5	-854.5
10A	3057.8	2662.2	-395.7	32.0	23.8	-8.16
10B	3174.5	2734.8	-439.7	34.7	17.0	-17.7
Mean	1441.8	1092.8	-348.9	585.4	496.9	-88.5
SD	1380.8	1265.3	249.7	535.0	502.2	663.1
Exclusion of anomalous result in patient 10						
Mean	604.6	290.0	-314.5	861.4	735.1	-126.3
SD	609.6	298.7	314.4	415.1	439.6	852.7

Appendix 8)

Tables demonstrating change in apoptotic ratios as assessed using TUNEL assay between non-irradiated samples, control and irradiated samples in three patient groups classified according to level of apoptotic ratio in each of the patient irradiated samples: A) Low (<30%) (p = 0.948), B) Medium (30-50%) (p < 0.0001) and C) High (>50%) (p = 0.0281) (Numbers to 3 significant figures or 1 decimal point)

A) Low apoptotic ratio group				
	Apoptotic ratio (%)			
Patient sample	Control	Irradiated		
4A	14.3	5.85		
4B	35.1	30.0		
4C	9.24	25.2		
Mean	19.6	20.3		
Standard deviation	13.7	12.7		

B) Medium apoptotic ratio group				
	Apoptotic ratio (%)			
Patient sample	Control	Irradiated		
2A	4.92	3.67		
2B	1.45	41.3		
2C	12.3	46.5		
3A	20.5	29.5		
3B	2.33	33.0		
3C	31.5	86.5		
5A	6.32	39.0		
5B	14.6	44.3		
5C	18.1	66.7		
6A	68.2	56.1		
6B	70.3	27.2		
6C	56.7	57.9		
7A	15.1	54.0		
7B	9.16	65.8		
7C	4.79	21.4		
7D	14.3			
10A	12.5	73.9		
10B	10.8	15.1		
10C	21.6	33.8		
10D	16.8	75.9		
11A	1.13	56.6		
11B	41.1	48.9		
11C	14.0	42.1		
11D	11.1	36.1		
Mean	20.0	45.9		
Standard deviation	19.8	20.3		

C) High apoptotic ratio group			
	Apoptotic ratio (%)		
Patient sample	Control	Irradiated	
1A	27.9	100	
1B	13.7	64.3	
1C	25.1	28.8	
1D		24.4	
1E		100	
1F		6.99	
8A	13.1	96.2	
8B	31.8	40.0	
8C	49.1	100	
8D	36.6	33.6	
9A	22.0	100	
9B	63.9	24.5	
9C	33.3	17.5	
9D	27.0	93.3	
Mean	31.2	59.3	
Standard deviation	14.9	37.3	

Appendix 9)

Tables demonstrating change in apoptotic ratios as assessed using M30 immunohistochemical assay between non-irradiated samples, control and irradiated samples in three patient groups classified according to level of apoptotic ratio in each of the patient irradiated samples: A) Low (<15%) (p = 0.117), B) Medium (15-30%) (p = 0.2003) and C) High (>30%) (p < 0.0001)

A) Low apoptotic ratio group		
	Percentage area undergoing apoptosis (%)	
Patient sample	Control	Irradiated
2A	10.5	7.93
2B	6.57	11.0
2C	18.9	11.6
4A	2.49	12.8
4B	2.43	11.7
4C	0.500	5.42
5A	1.30	129.7
5B	3.25	325.0
5C	4.53	452.7
7A	0.188	14.0
7B	14.0	1.35
7C	1.55	22.8
7D	5.87	11.7
9A	26.2	17.2
9B	22.5	17.0
9C	42.5	3.72
9D	24.2	1.46
Mean	11.0	62.2
Standard deviation	12.0	128.4

(Numbers to 3 significant figures or 1 decimal point)

B) Medium apoptotic ratio group		
	Percentage area undergoing apoptosis (%)	
Patient sample	Control	Irradiated
1A	0.932	2.05
1B	1.56	25.5
1C	1.30	23.5
3A	22.6	36.5
3B	26.4	25.4
3C	32.0	7.34
11A	2.00	2.37
11B	0.791	7.30
11C	4.17	12.0
11D	17.8	60.5
Mean	11.0	20.2
Standard deviation	12.4	18.3

C) High apoptotic ratio group		
	Percentage area undergoing apoptosis (%)	
Patient sample	Control	Irradiated
6A	1.32	35.6
6B	3.40	26.7
6C	1.45	43.0
6D	1.64	
8A	0.208	22.8
8B	0.899	22.3
8C	1.76	46.2
8D	3.17	29.1
10A	20.8	54.2
10B	18.4	24.8
10C	25.0	42.8
10D	20.1	38.3
Mean	8.18	35.1
Standard deviation	9.68	10.7

Appendix 10)

A) Table illustrating the compounds differentially expressed in the effluent from the irradiated tissue collected prior to irradiation and post irradiation (p < 0.0001).

'Compound' (retention time <i>m/z</i>	Anova (p)
value)	
1.19_236.1898m/z	1.1102230246251565E-16
1.14_192.1632m/z	1.6653345369377348E-15
20.44_261.1882m/z	2.0329293803911241E-12
18.61_277.1826m/z	3.2348568268503186E-12
1.58_236.1886m/z	1.1133427513243532E-11
1.55_192.1625m/z	1.2169343310830527E-10
2.08_263.2020m/z	2.9064217699215078E-09
18.89_223.1211m/z	2.9896797260065E-09
19.77_261.1852m/z	4.7063302144323416E-09
4.30_295.1556m/z	1.8570295745590215E-08
20.34_259.1691m/z	1.1360152163408799E-07
0.85_131.1205m/z	7.2092254665978572E-07
14.25_216.0708m/z	1.2026559611522103E-06
19.76_313.1454m/z	1.8714740066538837E-06
16.78_242.0840m/z	2.9120765151491312E-06
1.70_280.2184m/z	3.0106772626048439E-06
16.09_409.2483m/z	5.2577025786426645E-06
18.76_594.8647m/z	1.3523362970113695E-05
14.23_217.0670m/z	2.5197980283442334E-05
18.34_564.3765m/z	4.841194158811124E-05
18.24_763.5015m/z	4.858193484413853E-05
1.20_491.1903m/z	6.535408646557439E-05
18.79_613.0403m/z	8.7875061605458E-05
21.81_325.2192m/z	9.450221073115106E-05
20.27_372.2342m/z	9.9915448053655709E-05
17.86_524.3409m/z	0.00013090546782701384
19.29_352.9914m/z	0.045498435527664638
28.50_442.3094m/z	0.057423482625271194

B) Table illustrating the compounds differentially expressed in the effluent from the irradiated tissue collected prior to irradiation and post irradiation (p < 0.0001).

'Compound' (retention time <i>m/z</i>	Anova (p)
value)	
28.50_442.3094m/z	1.7022227741469997E-05
17.86_524.3409m/z	4.0699202356275777E-05
19.29_352.9914m/z	5.1974887623207344E-05