Predicting the Response to Radiotherapy for Rectal Cancer

Dr Jesse Fischer

A thesis submitted

for the degree of

Master of Medical Science

At

the University of Otago,

Christchurch, New Zealand

30 March 2019

Acknowledgments

Sincere gratitude goes to the following, without whom this thesis would not have been possible:

Associate Professor Tim Eglinton and Professor Frank Frizelle for their incredible support, guidance and inspiration during my surgical training.

Professor Mark Hampton and his team for welcoming me into the Centre for Free Radical Research. Dr Andree Pearson and Dr Paul Pace for technical and scientific advice, and Dr Nick Magon for performing the carbonyl ELISA in chapter 4.

Helen Morrin and Lucia Sinclair of the Cancer Society Tissue Bank and endoscopy suite staff at Christchurch Hospital for assistance in collecting specimens for the study in chapter 4.

Dr Simon Richards for his assistance with article selection for the systematic review in chapter 1.

Colorectal Surgical Society of Australia and New Zealand (CSSANZ) for awarding me the Medtronic Research Fellowship, and the CSSANZ Foundation for additional project funding.

The patients who donated tissue for use in the study described in chapter 4. As with all clinical research, you are the whole reason and means for this work.

My wife Andrea and daughters Tahlia, Helena and Esme - for always reminding me what matters most.

Peer reviewed presentation arising from work described in this thesis

Peroxiredoxins as markers of oxidative stress: potential predictors of response to radiotherapy for rectal cancer? Fischer J, Eglinton TW, Pearson AG, Pace PE, Frizelle FA, Hampton MB. Society for Free Radical Research Australasia Conference, 8-12 December 2018, Auckland University, Auckland. (*Oral and poster presentation*).

Peer reviewed publication arising from work described in this thesis

Fischer J, Eglinton TW, Frizelle FA, Hampton MB. Peroxiredoxins in Colorectal Cancer: Predictive Biomarkers of Radiation Response and Therapeutic Targets to Increase Radiation Sensitivity? Antioxidants (Basel). 2018 Oct 5;7(10). pii: E136. doi: 10.3390/antiox7100136.

Award resulting from the work described in this thesis

Society for Free Radical Research Australasia (University of Otago) Travel Award. Awarded to facilitate presentation of research at the society conference 8-12 December 2018.

Abstract

Aim

To investigate predictors of pathological response to radiotherapy for rectal cancer by reviewing the evidence, exploring clinicopathological predictors in a local cohort and investigating markers of oxidative stress as novel biomarker predictors.

Method

A systematic review of the literature was undertaken. A retrospective study of patients treated with neoadjuvant chemoradiotherapy (nCRT) and total mesorectal excision at Christchurch Hospital 2013-2018 was performed by reviewing patients clinical records. A literature review of the role of antioxidants including peroxiredoxins on the effect of ionising radiation was performed. A prospective observational pilot study was then performed investigating markers of oxidative stress including peroxiredoxin oxidation status and protein carbonyls as novel predictors of the response to radiotherapy for rectal cancer.

Results

Based on currently available evidence the predictors of pathological response to nCRT for rectal cancer with the most clinical utility currently are clinical T and N stage, tumour size and CEA level. In a cohort of 164 patients treated with nCRT and total mesorectal excision there was a pathological complete response (pCR) rate of 14.6%; shorter tumour length on MRI and lower clinical N stage were independent predictors of pCR on logistic regression. Higher BMI, anterior or circumferential tumours and lower haemoglobin were independent predictors of minimal-poor response as determined by tumour regression grade.

The markers of oxidative stress peroxiredoxin 2 and peroxiredoxin 3 percentage oxidation and protein carbonyl levels did not predict response to radiotherapy for rectal cancer in seven patients treated with nCRT, of which two patients experienced a pCR and one patient experienced a complete clinical response.

Conclusion

The pathological response to radiotherapy for rectal cancer is complex and numerous factors appear to play a role. Predictors can be classified as clinicopathological, biomarker or

radiological predictors. Although there are several clinical predictors of use currently, none have a high predictive ability. Neither peroxiredoxin oxidation status or protein carbonyls appear to predict the response to radiotherapy for rectal cancer. A composite scoring system comprised of clinicopathological, biomarker and radiological factors is most likely to yield a useful predictive tool in the future.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

$\%Prx_{ox}$	Percentage oxidation of peroxiredoxin
5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
BMI	Body mass index
cCR	Complete clinical response
CEA	Carcinoembryonic antigen
CI	Confidence interval
CR	Complete response
CRC	Colorectal cancer
CRT	Chemoradiotherapy
CT	Computed tomography
DNA	Deoxyribonucleic acid
dTRG	Dichotomised tumour regression grade
ELISA	Enzyme linked immunosorbent assay
GR	Good response
IHC	Immunohistochemistry
IR	Ionising radiation
LARC	Locally advanced rectal cancer
LVI	Lymphovascular invasion
miRNA	Micro-ribonucleic acid
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
n	Number of participants
NEM	N-ethylmaleimide
nCRT	Neoadjuvant chemoradiotherapy
NS	Not statistically significant
OR	Odds ratio
PBS	Phosphate buffered saline
pCR	Pathological complete response
PET	Positron emission tomography
PNI	Perineural invasion
PR	Pathological response

Prx	Peroxiredoxin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCRT	Short course radiotherapy
SD	Standard deviation
SEM	Standard error of the mean
TILs	Tumour-infiltrating lymphocytes
TME	Total mesorectal excision
TNM	Tumour node metastasis
TRG	Tumour regression grade
W&W	Watch-and-wait

1 Introduction and Systematic Review

1.1 Introduction

Colorectal cancer (CRC) is the third most common cancer in the Western world and the second most common cancer in New Zealand, where approximately 3000 new cases are diagnosed and 1200 deaths occur annually from the disease (1). Approximately one-third of cases of CRC are rectal in origin. Colon cancer is generally treated by surgical resection +/- adjuvant chemotherapy, usually indicated for lymph node metastases or occasionally for advanced T stage tumours. Rectal cancer holds specific treatment challenges compared with colon cancer, often requiring specialist surgical care and multi-disciplinary treatment.

Bowel cancer screening programs reduce death rates from CRC (2), and the introduction of screening is currently taking place in New Zealand. This means a larger proportion of patients are likely to be diagnosed with curable disease due to earlier diagnosis. There is also evidence of a rising incidence of CRC in people under 50 years of age, especially rectal cancer in males under 50 years (3). These two factors, combined with advances in treatment, mean a greater proportion of long-term survivors may live with the morbidity of rectal cancer treatment for decades. This has major implications for the selection of treatment strategies.

1.1.1 Modern Management of Rectal Cancer

Traditionally surgery has been seen as the only curative option for rectal cancer but it carries significant risks including a 1-2% in-hospital mortality rate, 31% major complication (Clavien-Dindo grade 3-4) rate and an approximately 25% chance of a permanent stoma (4), as well as the risk of significant post-operative bowel dysfunction known as low anterior resection syndrome (LARS) (5).

Historically, local recurrence in the pelvis was a major problem in the management of rectal cancer and local recurrence rates of 25% could be expected (6). Pre-operative radiotherapy and TME have significantly reduced this risk (7, 8) and are now standard of care for many patients. The standard curative treatment options for localised rectal cancer are surgical resection alone or surgical resection after pre-operative (neoadjuvant) or post-operative radiotherapy. An alternative strategy of non-operative management (known as ‘watch-and-wait’ management) is starting to emerge as a viable alternative in appropriately selected patients (9). Adjuvant chemotherapy is usually indicated for lymph node metastases and can

be considered after resection irrespective of neoadjuvant therapy received. Chemotherapy has been shown to improve survival by delaying or preventing systemic recurrence (10).

The management of patients with distant metastases is usually palliative, although curative treatment remains a possibility when all disease is resectable. Treatment strategies become complex for such patients and should be decided in a multi-disciplinary setting.

1.1.1.1 Radiotherapy for Rectal Cancer

Local recurrence (LR) rates are reduced when radiotherapy is used in addition to surgery for rectal cancer, but there is no definitive evidence of survival benefit from randomised trials (11, 12). Radiotherapy is therefore given to improve patients' quality of life, and indeed pelvic recurrence of malignancy is a morbid event. This must be balanced against the morbidity of the treatment; radiotherapy is therefore used selectively for patients with rectal cancer.

Indications for neoadjuvant radiotherapy include locally advanced rectal cancer (LARC), usually defined as those tumours with a clinical T stage of T3 or greater by the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) staging system, radiological suspicion of mesorectal lymph node involvement, a threatened circumferential resection margin (CRM), or tumours close to the anorectal margin traditionally necessitating abdomino-perineal resection. Common contraindications to radiotherapy include prior pelvic radiation or patient factors such as co-morbidity and frailty.

Radiotherapy is given with or without low-dose chemotherapy as a radiosensitiser, and may be given pre-operatively or post-operatively. Two main neoadjuvant treatment strategies exist for rectal cancer, termed 'short-course' or 'long-course'. 'Short-course' radiotherapy (SCRT) consists of 25 Gray delivered in five fractions over five days with rectal resection usually performed within 7-10 days. Chemotherapy is not given concurrently. 'Long-course' (nCRT) regimens vary but a typical regimen consists of 45 Gray whole pelvis irradiation and a 5.40 Gray boost to the tumour, delivered in 28 fractions over five and a half weeks. It is usually given with fluoropyrimidine-based chemotherapy (i.e. oral capecitabine or infusional 5-fluoruracil) unless there are contraindications. Fluoropyrimidine therapy has been demonstrated to improve treatment response (13); there is equivalence between capecitabine and 5-FU (14). The interval after nCRT to surgery has historically been at least 6

weeks but there is evidence a longer interval improves pathological response (15), with some surgeons now waiting 12 weeks or longer post-nCRT prior to operating.

There is evidence to support both SCRT and nCRT regimens as neoadjuvant therapy for rectal cancer. SCRT reduces LR compared with no neoadjuvant treatment (16), and an Australasian randomised trial found no significant difference in LR between SCRT and nCRT in T3N0-2M0 rectal cancer patients (17). nCRT may have a greater reduction in LR in some circumstances (17), and there is a trend to favour nCRT when an optimal response is necessary to increase the chance of an R0 resection (for example a threatened circumferential resection margin) (18), particularly in younger fitter patients who will tolerate the additional therapy. pCR rarely occurs in SCRT with early surgery (rates 0-1.7%), but with an interval to surgery of 4-6 weeks this increases to rates almost comparable with nCRT (11.8-15%) (19-21). There is significant variation in the ratio of SCRT to nCRT regimens used across New Zealand and the world.

Post-operative radiotherapy is an alternative to pre-operative radiotherapy, although the evidence to support it is not as strong and as a result it tends to be used when it was not possible to deliver pre-operative radiotherapy (22). Evidence suggests the reduction in LR is greater with pre-operative treatment (13% compared with 6%) and late radiation toxicity is less (23); pre-op SCRT has been found to have lower LR rates than post-operative chemoradiotherapy (CRT) (24). Although the optimal regimen remains controversial, it is apparent that radiotherapy should be given pre-operatively if possible.

Radiotherapy has significant morbidity associated with it, including early and late effects. Early toxicity includes skin erythema, diarrhoea and pain. Late effects include bowel obstruction, altered bowel habit, faecal incontinence and faecal urgency, and sexual dysfunction (25). There are specific socioeconomic implications for patients receiving radiotherapy for rectal cancer in New Zealand. Radiotherapy services are concentrated in major centres, with two radiotherapy centres in the South Island servicing that vast geographical area. Patients from rural areas can spend up to six weeks away from home in order to receive radiotherapy treatment and socioeconomic barriers may prevent some patients from receiving optimal treatment. Maori are slightly more likely to live rurally, and this additional barrier could perpetuate health disparities for Maori patients with rectal cancer.

1.1.1.2 Pathological Response to nCRT

There is a highly variable pathological response to nCRT for rectal adenocarcinoma; up to 20% of patients have a pathological complete response (pCR), 20-38% demonstrate either tumour progression or negligible regression and the remainder experience a significant (>50%) but incomplete response (26). A pCR occurs when no residual adenocarcinoma can be identified in the resection specimen after total mesorectal excision (TME) following neoadjuvant treatment, designated ypT0N0 by the AJCC staging system. This is recognised as a good prognostic factor and a survival advantage has been demonstrated in this group compared to those without pCR (27). It is probable that those who obtain a moderate response also obtain some benefit, especially if a previously threatened CRM is no longer threatened. Patients who experience negligible regression are unlikely to obtain benefit from neoadjuvant radiotherapy, and some patients may even be harmed as local and distant control of malignancy may be compromised.

There are numerous systems described to assess the histological tumour regression grade (TRG) of rectal cancer following radiotherapy (28), the most widely used systems are presented Table 1.1. Tumour regression grading systems often consider only disease remaining in the bowel wall, not lymph node involvement. Tumour regression grading is generally accepted as the optimal outcome measure of disease response although no system is perfect; down-staging is likely to be a less reliable measure of disease regression given sub-optimal accuracy of clinical staging.

Table 1.1. Tumour regression grade systems (modified from reference (28))

	Dworak	**Mandard**	**Ryan**	**AJCC**
Complete regression	No tumour cells (TRG 4)	No residual cancer cells (TRG 1)	No viable cancer cells, or single cells, or small groups of cancer cells (TRG 1)	No viable cancer cells (TRG 0)
Near complete regression	Very few tumour cells (TRG 3)	Rare residual cancer cells (TRG 2)	-	Single or small groups of tumour cells (TRG 1: moderate response)
Moderate regression	Dominantly fibrotic changes with few tumour cells or groups (TRG 2)	Predominant fibrosis with increased number of residual cancer cells (TRG 3)	Residual cancer outgrown by fibrosis (TRG 2)	Residual cancer outgrown by fibrosis (TRG 2: minimal response)
Minimal regression	Dominant tumour mass with obvious fibrosis (TRG 1)	Residual cancer outgrowing fibrosis (TRG 4)	Significant fibrosis outgrown by cancer, or no fibrosis with extensive residual cancer (TRG 3)	Minimal or no tumour cells killed (TRG 3: poor response)
No regression	No regression (TRG 0)	No regressive change (TRG 5)	-	-

Theoretically the ability to predict a poor response to nCRT could allow omission of the same if lack of benefit is also demonstrated in clinical studies, and patients could proceed directly to surgery. There is good evidence that pCR is associated with lower rates of local and distant recurrence (27); the impact of TRG on local recurrence has not been well studied but there is evidence that TRG is associated with local recurrence rates (29). Conversely, the ability to predict a complete clinical response (cCR) may support a trial of watch-and-wait (W&W) management.

1.1.1.3 Watch-and-Wait Treatment of Rectal Cancer

There is currently a significant shift occurring in rectal cancer treatment, with an increasingly prevalent view towards organ preservation using a W&W strategy after CRT as a viable alternative to surgical resection. The use of W&W relies on a cCR being achieved. This development may be the single biggest advancement in rectal cancer treatment in decades.

CRT is traditionally used in the neoadjuvant setting, although in some cases CRT is now being delivered with curative intent. A cCR occurs when there is no identifiable tumour remaining (intra-luminally or extra-luminally) on clinical examination, endoscopic or imaging assessment.

Major advancement in W&W treatment of rectal cancer is largely due to the work of Habr-Gama and colleagues in Brazil who first published their landmark paper in 2004 (30), but W&W now has significant uptake in centres of excellence worldwide (31, 32). The omission of early surgery may risk missing the chance of cure for loco-regionally confined malignancy, and death from rectal cancer progression may result. The benefits of omitting surgery are that the morbidity and mortality risks of rectal resection are avoided. Embarking on W&W is a complex decision that must consider patient and disease factors; rectal resection (with or without neoadjuvant treatment) remains the standard of care for rectal cancer at the current time. W&W should only be performed with input from a specialist centre with multi-disciplinary agreement and extensive discussion with the patient, ideally in a clinical trial or international registry.

Organ preservation is standard practice for some cancers including bladder and breast cancer, but these have major differences to rectal cancer. Breast cancer in particular has experienced a progressive reduction in the aggressiveness of surgery over decades. Breast-conserving surgery and axillary node sampling is now routine for many patients with breast cancer; the highly morbid total mastectomy and axillary node dissection which was performed routinely historically is now reserved for selected cases. The avoidance of high-morbidity surgery should be the goal of all surgeons, provided oncological outcomes are not compromised.

The role of additional chemotherapy after a cCR to CRT remains a controversial but developing area. Along with the rise of W&W we are also seeing alternative treatment strategies including total neoadjuvant treatment (CRT and chemotherapy) with significant increase in rates of pCR (33), with or without deferral of surgery if a cCR is achieved.

W&W management of rectal cancer is a major developing field but there are still major barriers that need to be overcome if W&W is to become standard of care for a significant proportion of rectal cancer patients. These include accurately predicting which patients are likely to respond to CRT (optimising patient selection), optimising treatment response to CRT

by delivering the most effective treatment regimen, accurately assessing the clinical response to CRT, closely monitoring for early identification of loco-regional recurrence and effectively treating recurrence with salvage surgery while avoiding additional risk of mortality and morbidity over early resection.

1.1.1.4 Summary and Outline of Thesis

This thesis is focused on predicting the pathological response to nCRT for rectal cancer. The pathological response to nCRT is highly variable, with pCR rates ranging from 10-40%, and poor response rates of up to 40% (26). The exact reasons for differing response rates are unclear but is likely a combination of patient and disease characteristics, treatment regimens and outcome measures such as methods used to grade response. Predicting response is a critically important aspect of cancer treatment as it allows the avoidance of morbid and resource-demanding treatment in patients unlikely to benefit. There are clinical benefits to predicting good and bad responders to any treatment, but surgery and radiotherapy for rectal cancer carries significant morbidity and mortality risk only deemed acceptable in the pursuit of prolonging quantity or quality of life. The acceptability of such treatment in patients predicted to have low rates of response would be seriously questioned. An understanding of the impact of different predictors on pathological response would allow an increased understanding of the reasons for the different response rates observed.

The remainder of chapter one includes a comprehensive systematic review of predictors of pathological response to nCRT for rectal cancer. This demonstrates current methods of prediction are blunt tools at best, and better predictors are needed to effectively personalise treatment.

Chapter 2 reports a retrospective study examining clinical predictors in a five year cohort of patients treated with nCRT and TME at Christchurch Hospital, and describes an attempt to develop a simple clinical scoring tool based on predictors identified. Limited predictive ability of clinical predictors lead on to theorisation of a novel biomarker approach in chapter 3, a literature review exploring the role of oxidative stress in tumour cell death from radiotherapy and the potential role of endogenous antioxidants, specifically the peroxiredoxin family of thiol-dependent proteins, in predicting response. A prospective pilot study assessing selected biomarkers of oxidative stress and mitochondrial function using laboratory techniques novel in translational research is reported in Chapter 4.

Chapter 5 is a summary of findings from this thesis and discussion of potential future strategies to predict the response to CRT for rectal cancer across all categories of predictors, including clinical, biomarker and radiological predictors.

1.2 Predicting Pathological Response to Neoadjuvant Chemoradiotherapy for Rectal Cancer: a Systematic Review of the Literature

1.2.1 Introduction

Within the current paradigm of rectal cancer treatment, a reliable predictor of response to nCRT for rectal cancer would allow for better selection of patients to receive nCRT prior to rectal resection. If poor response was predicted it could be safely omitted, and the patient would be best to proceed directly to surgery. If a good or even complete clinical response was likely nCRT could be more strongly recommended, including for early tumours not routinely treated with nCRT in current clinical practice (e.g. stage I rectal cancer). In the evolving field of a W&W strategy for rectal cancer, predicting response to radiotherapy would allow early selection of patients who could embark on W&W. Pathological complete response is a robust and clinically important outcome measure and has been a major focus of the literature so far, but prediction of complete responders is arguably less important than prediction of poor responders in the current environment where nCRT is the standard of care for LARC and would likely be offered if a complete or moderate response was expected. Those with a predictably poor response are the patients in whom omitting nCRT might be most advantageous.

There has been a large and rapidly increasing volume of research attempting to identify predictive markers of pCR in rectal cancer. Predictive markers for pathological response (PR) are classified into three groups: clinicopathological, radiological and biomarkers. A previous systematic review by Ryan et al provided a summary of the evidence for predictive markers for pCR up to 2015 (26) but since this review was published the literature has expanded rapidly and a follow-up review is appropriate. Given the potential benefit in identifying poor responders and the limitations of focusing solely on pCR, this review includes all articles assessing pathological response as an outcome rather than exclusively pCR.

1.2.2 Method

A PubMed, Embase and Medline search was performed for 2008-2018. The PubMed search was based on text words rectal cancer, pathological response and neoadjuvant chemoradiation, filtered for humans and English language. An Embase and Medline search was constructed for rectal cancer.mp. or Rectal Neoplasms/ AND Neoadjuvant Therapy/ or Chemoradiotherapy.mp. AND path* response*.tw. limited to English and Humans, excluding review articles. The initial search criteria were intentionally broad and included an outcome of pathological response rather than exclusively pCR. Inclusion criteria required an outcome of pathological tumour response measured by either pCR or tumour regression grade (TRG), pre-treatment predictors examined in relation to pathological tumour response, treatment with fluoropyrimidine-based concurrent neoadjuvant chemoradiotherapy for rectal adenocarcinoma. TRG as a categorical variable was acceptable provided the classification method was defined and based on a recognised TRG. Exclusion criteria included using down-staging as a sole measure of response to CRT, use of extended neoadjuvant chemotherapy, review articles and meta-analyses, and studies with less than 30 participants. The article selection process is outlined in Figure 1.1. The search strategy was finalised in consultation with a librarian to ensure the optimal strategy was used. The search process and article inclusion/exclusion was performed independently by JF and SR who then discussed discordance and agreed on exclusion or inclusion for each article where necessary. Mediation was not necessary.

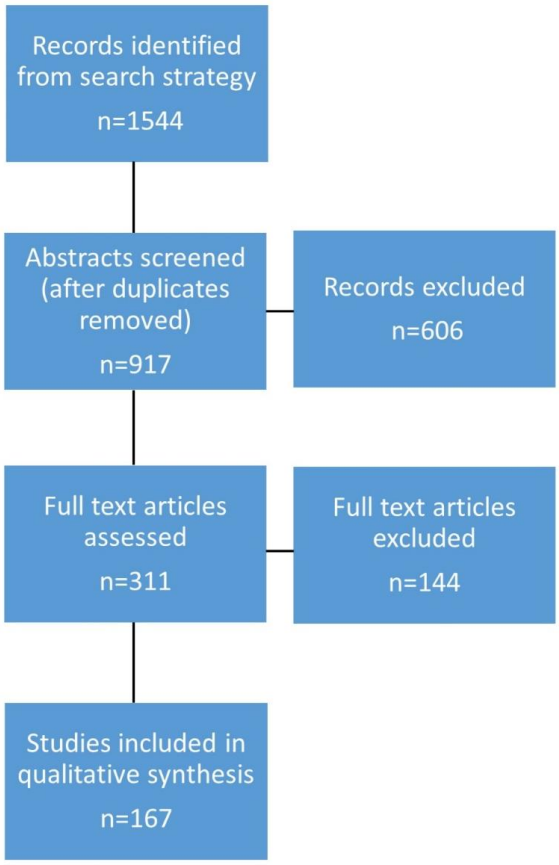

Figure 1.1. Article selection process for the systematic review

1.2.3 Results

167 articles were included in the qualitative synthesis. 56 articles were published subsequent to the last systematic review first published in 2015, demonstrating significant growth of literature in recent years. Outcome measures used in the literature for this review were limited to pCR, good response (GR) by dichotomised TRG (dTRG), and TRG as an ordinal variable.

Predictors were categorised as clinicopathological predictors, biomarker predictors or radiological predictors. Table 1.2 outlines sub-categories of predictors included in the review.

Table 1.2. Predictors of response to nCRT included in this systematic review.

Clinicopathological predictors	**Biomarker predictors**		**Radiological predictors**
Patient factors e.g. demographics, co-morbidity	***Tumour***	Protein expression (IHC)	CT
Histopathology		Gene methylation	MRI
Clinical examination findings		Cytogenetics	PET
Endoscopic findings	***Blood***	Serum peptides e.g. CEA	
Clinical TNM staging		Haematological parameters e.g. haemoglobin, lymphocyte	
	Tumour or blood	Gene sequencing including microarrays/gene signatures	
		Single nucleotide polymorphisms	
		mRNA	
		mi-RNA	

1.2.3.1 Clinicopathological Predictors

Clinicopathological predictors include clinical examination and endoscopic findings, histopathology reports and information routinely reported during radiological staging of LARC. This includes papers dedicated to clinicopathological predictors as well as data extracted from biomarker and radiological studies. Clinicopathological predictors were mostly investigated in retrospective studies. There were three notable database studies that were significantly larger than all other studies, those by Probst et al (34), Lorimer et al (35) and Al-Sukhni et al (36), with 18,113, 27,532 and 23,717 patients included respectively.

1.2.3.1.1 Patient Factors

Gender as a predictor of PR has been examined in a large number of retrospective studies which have shown no difference (37-48), although two large database studies found a small but significantly increased chance of pCR in females with an OR 1.24 (1.09-1.41, p=0.002) (34) and OR 1.12 (1.03-1.22, p=0.0078) (49).

Diabetes mellitus has been suggested as a negative predictor of pCR in a single retrospective study, with 0/17 diabetics achieving pCR compared with 21/93 (23%) non-diabetics (50), although Huh et al found no significant difference for pCR rate in diabetics on multivariate analysis (51). Increased body mass index (BMI) has been associated with poorer TRG (52) but several retrospective studies (n=242 to n=885) have reported no difference for pCR (37, 38, 48, 51).

1.2.3.1.2 Histopathology

Table 1.3 presents histopathological factors identified on pre-treatment biopsy. For differentiation, five of six studies found no significant difference while one study found poor differentiation was associated with a lower rate of pCR (53). Poor differentiation coupled with a T4 primary tumour predicted extreme unresponsiveness to nCRT in a Chinese population (54).

Probst et al found an OR of 0.15 (0.10–0.28, $p<0.001$) for pCR for mucinous tumours (34), consistent with a previous systematic review and meta-analysis (55). Two studies found a trend without statistical significance for poorer response if mucinous histology is present; there was a low incidence of mucinous tumours in those papers (54, 56). One study suggested higher response rate with signet ring type but this is a rare cancer and numbers were small (57); Probst et al found a trend only (34). A single study suggested pCR is unlikely if tumour budding is present as a pCR was achieved in 0/18 patients with tumour budding (58), but the result was not statistically significant and tumour budding has rarely been reported for histopathology of biopsies historically. Few studies have examined lymphovascular invasion and perineural invasion, but Li et al found no association with pCR (59).

Table 1.3. Histopathological predictors of pathological response to nCRT for rectal cancer.

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
Differentiation categories						
Grade 1-3	215	TRG	NS	-	Retrospective	(56)
Well/moderate/poor	274	pCR	NS	Y	Retrospective	(60)
Well/moderate/poor	121	dTRG	NS	-	Retrospective	(39)
Well/moderate/poor	168	pCR	NS	-	Retrospective	(41)
Well/moderate/ poor/undifferentiated	23,747	pCR	Poor differentiation OR 0.78 (0.63-0.96) c.f. well differentiated, p=0.002	Y	Retrospective	(53)
Well-differentiated/ not well differentiated	86	dTRG	NS	Y	Retrospective	(45)
Mucinous histology	18,113	pCR	OR 0.15 (0.10, 0.28), p<0.001	Y	Retrospective	(34)
	248	pCR	NS	Y	Retrospective	(57)
	168	pCR	NS	-	Retrospective	(41)
	96	pCR	NS	-	Retrospective	(54)
	215	TRG	NS	-	Retrospective	(56)
Signet ring histology	248	pCR	RR 10.07 (2.47-41.10), p=0.001.	Y	Retrospective	(57)
	18,113	pCR	NS	Y	Retrospective	(34)
Tumour budding	89	pCR; TRG	NS	-	Retrospective	(58)

1.2.3.1.3 Endoscopic and Examination Findings

Table 1.4 presents endoscopic and examination findings investigated as predictors, the main findings of which are summarised in the following text.

1.2.3.1.3.1 Distance to the Anal Verge

Distance to the anal verge has been investigated in 12 studies as both continuous and categorical variables. Three studies found a significant difference in response rate but a wide range of measures were used using different categorisation of distance (Table 1.4). The studies were in agreement that a greater distance is likely to be associated with better chance of GR but this evidence is moderately weak. Distance to the anal verge should be measured using rigid sigmoidoscopy (61), but is often assessed clinically using digital rectal examination or flexible endoscopy; it is hence likely to be variably recorded and of limited accuracy. Distance to the anorectal margin on MRI is a more reproducible measure of position in the rectum that may be routinely included in synoptic reports, although arguably less clinically important due to limited accuracy of this measure also.

1.2.3.1.3.2 Tumour Size

Tumour size has been reported as a continuous and categorical variable, reported in eight studies. One study identified it as a predictor as a continuous variable with larger tumours less responsive (62), one suggested >5cm size is predictive of not achieving pCR (63). Although this evidence is mixed, the largest review of 23,747 patients found increasing size a significant predictor of poor response (53). Based on this evidence, increasing size appears associated with poorer response.

Tumour site has been investigated in one study which found no difference in pCR for anterior compared with posterior tumours (41). Guedj found tumours are more often flat in responders but was the only study to assess this (64); flatness is an unusual description and may be a surrogate of total tumour volume. Absence of macroscopic ulceration was a predictor of pCR in one study (51). Weak evidence supports tumour mobility as associated with better pathological response (42, 65). Two out of four studies of circumferentiality found no association with pCR (41, 42), with only one suggesting non-circumferentiality predicts pCR (51) and one suggesting non-circumferentiality predicts GR (65).

Table 1.4. Endoscopic and examination predictors of pathological response to nCRT for rectal cancer

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
DTAV						
<4cm, 4-6cm, 6-8cm, 8-10cm, >10cm	827	pCR	4-6cm OR 2.54 (1.36-4.75), 6-8cm OR 2.55 (1.37-4.74) c.f. <4cm, p=0.008	Y	Prospective	(66)
≤6 or >6cm	98	dTRG	NS	Y	Prospective	(65)
>8cm or ≤ 8cm	99	pCR	NS	-	Retrospective	(63)
>5cm or ≤5cm	249	pCR	NS	-	Retrospective	(42)
>5cm or ≤5cm	260	pCR	>5cm OR 3.82 (1.60-8.70), p=0.02	Y	Retrospective	(67)
≥5cm or <5cm	95	pCR	NS	-		(59)
Low/medium/high	168	pCR	NS	-	Retrospective	(41)
Continuous variable	242	pCR	NS	-	Retrospective	(38)
Continuous variable	274	pCR	NS	-	Retrospective	(60)
Continuous variable	885	pCR	OR 1.07 (1.01-1.15), p=0.04	Y	Retrospective	(37)
Continuous variable	620	pCR	NS	Y	Retrospective	(48)
Macroscopic ulceration (absence of)	391	pCR	OR, 6.70 (2.04-22.07), p=0.002	Y	Retrospective	(51)
Circumferentiality						
Non-circumferentiality	391	pCR	OR 3.21 (1.38-7.50), p=0.007	Y	Retrospective	(51)
Circumferentiality	98	dTRG	Increased circumferentiality had increased risk of poor	Y		(65)

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
			response, HR 2.39 (1.14-3.40), p=0.019			
Circumferentiality	168	pCR	NS	-	Retrospective	(41)
Circumferentiality	249	pCR	NS	-		(42)
Tumour size						
<1cm/ 1–2cm/ 2–3cm/ 3–4cm/ 4–5cm/ >5cm	23,747	pCR	<1cm = reference, p<0.001 OR 1.22 (0.87–1.71)/ 2.16 (1.59–2.95)/ 3.16 (2.33–4.29)/ 3.13 (2.30–4.25)/ 3.07 (2.27–4.14)	Y	Retrospective	(53)
<3.5cm/ 3.5-7cm/ >7cm; Continuous variable	297	pCR	NS for categorical analysis; pCR increased with decreasing tumour size OR 1.315 (1.018–1.698), p=0.036	Y	Retrospective	(68)
>5cm/ ≤5cm	99	pCR	≤5cm OR 0.25 (0.1-3.44), p=0.035	Y	Retrospective	(63)
>5cm/ ≤5cm	121	dTRG	NS	-	Retrospective	(39)
> 4/ ≤ 4	249	pCR	NS	Y	Retrospective	(42)
Continuous variable	391	pCR	NS	Y	Retrospective	(51)
Continuous variable	168	pCR	NS	-	Retrospective	(41)
Continuous variable	67	pCR	OR 0.65 (0.45-0.94), p=0.021	Y	Retrospective	(62)

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
Tumour mobility	98	dTRG	HR 2.65 (1.43-4.90), p=0.002	Y	Retrospective	(65)
	168	pCR	NS	-	Retrospective	(41)
	249	pCR	OR 2.78 (1.14-6.78), p=0.024	Y	Retrospective	(42)

DTAV = distance to the anal verge

1.2.3.1.3.3 Clinical TNM Staging

Table 1.5 presents studies that have investigated clinical TNM stage as a predictor.

Clinical tumour stage (cT) has been examined often and only three studies have demonstrated significant associations with pathological regression, however these included the two studies that were largest by far (49, 53) and both found T4 tumours were approximately half as likely to achieve pCR compared with T1-T3 tumours. These findings were supported by a third smaller study which compared T4 to T3 and found a similar effect, albeit with a small sample size and wide confidence interval (69). pCR appears significantly less likely for T4 tumours but the difference in response for lower T stage tumours does not appear as great.

Clinical nodal stage (cN) was assessed either as a binary variable for nodal positivity (N0 vs. N1/2) or by categorical N stage (N0 vs. N1 vs. N2). Nodal positivity has been associated with poorer TRG (56, 69, 70) and lower rate of pCR (68, 71). Only one study found no difference for dTRG (45) but four studies found no difference for pCR. As an ordinal variable four studies found no difference and four studies found increasing cN stage was associated with a decreasing pCR rate, including Lorimer et al (49) and Al-Sukhni et al (53) with an OR of 1.6 for pCR in N0 vs. N2 and a 30% reduction in pCR for N2 stage compared with N0 respectively. Nodal involvement appears to be a predictor of poor pathological response and this difference may be greatest for N2 disease.

Table 1.5. Clinical TNM stage as a predictor of pathological response to nCRT for rectal cancer

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
Clinical T stage						
cT1/T2/T3/T4	27,532	pCR	T4 = reference, p<0.0001 T1: OR 2.81 (1.80–4.40) T2: OR 2.72 (2.09–3.55) T3: OR 1.90 (1.55–2.33)	Y	Retrospective	(49)
cT1/T2/T3/T4	23,747	pCR	T1 = reference, p=0.002 OR for T2: OR 0.84 (0.63-1.12) T3: OR 0.80 (0.62-1.04) T4: OR 0.57 (0.42-0.78)	Y	Retrospective	(53)
cT1/T2/T3/T4	33	pCR	NS	-	Retrospective	(43)
cT1/T2/T3/T4	469	pCR	NS	Y	Retrospective	(40)
cT2/T3/T4	297	pCR	NS	-	Retrospective	(68)
cT2/T3/T4	86	dTRG	NS	Y	Retrospective	(45)
cT2/T3/T4	168	pCR	NS		Retrospective	(41)
cT2 or cT3 /cT4	122	pCR	NS	Y	Retrospective	(71)
cT2 or cT3 /cT4	274	pCR	NS	-	Retrospective	(60)
cT3/4	96	pCR	NS	-	Retrospective	(72)

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
cT3/ cT4	148	Good response (dTRG)	cT3 OR 2.762 (1.11-6.36), p=0.039	Y	Retrospective	(69)
cT3/cT4	98	dTRG	NS	Y	Prospective	(65)
cT =3/MRF- / cT =3/MRF+ / cT =4 / cT=1–2	620	pCR	NS	Y	Retrospective	(48)
Clinical N stage						
cN+ or cN0	215	TRG	cN+ poorer TRG, p=0.008	N	Retrospective	(56)
cN+ or cN0	148	Good response (dTRG)	cN0 OR 2.65 (1.14 – 6.52), p=0.034	Y	Retrospective	(69)
cN+ or cN0	609	Good response (dTRG)	cN0 OR 1.91 (1.23-2.95), p=0.004	Y	Retrospective	(70)
cN+ or cN0	122	pCR	cN+ negative predictor for pCR, with HR 3.701 (1.03 – 13.26), p=0.044	Y	Retrospective	(71)
cN+ or cN0	297	pCR	cN0 OR 4.4 (1.01 - 19.02), p=0.048	Y	Retrospective	(68)
cN+ or cN0	96	Good response (dTRG)	cN0 OR 1.98 (1.04–4.80), p=0.005	Y	Retrospective	(72)

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
cN+ or cN0	86	dTRG	NS	Y	Retrospective	(45)
cN+ or cN0	168	pCR	NS		Retrospective	(41)
cN+ or cN0	274	pCR	NS	-	Retrospective	(60)
cN+ or cN0	885	pCR	NS	Y	Retrospective	(37)
cN+ or cN0	391	pCR	NS	Y	Retrospective	(51)
cN0/N1/N2	242	pCR	NS	Y	Retrospective	(38)
cN0/N1/N2	99	pCR	NS	Y	Retrospective	(63)
cN0/N1/N2	33	pCR	NS	-	Prospective	(43)
cN0/N1/N2	469	pCR	NS	Y	Retrospective	(40)
cN0/N1/N2	55	pCR	cN lower in pCR, p = 0.002	N	Retrospective	(73)
cN0/N1/N2	620	pCR	cN0 = reference, p=0.04 cN1: OR 3.42 (1.03–11.32) cN2: OR 4.52 (1.32–15.55)	Y	Retrospective	(48)
cN0/N1/N2	23,747	pCR	cN0 = reference, p=0.014 cN2: OR 0.71 (0.56–0.89) cN1: OR 0.97 (0.88–1.07)	Y	Retrospective	(53)
cN0/N1/N2	27,532	pCR	cN2 = reference, p<0.0001 cN0: OR 1.62 (1.35–1.94) cN1: OR 1.41 (1.17–1.69)	Y	Retrospective	(49)

MRF+ = circumferential resection margin involved; **MRF-** = circumferential resection margin not involved

1.2.3.2 Biomarker Predictors

Biomarkers have been extensively investigated with a large number of potential candidates identified. Biomarkers can be either tissue or blood based and have been previously categorised in Table 1.2.

1.2.3.2.1 Carcinoembryonic Antigen (CEA)

CEA as a predictor of pCR has been the subject of a systematic review and meta-analysis published in 2017 which concluded CEA was inversely correlated with probability of pCR (74). The evidence found in the current review is summarised in Table 1.6, and studies published subsequent to the meta-analysis appear unlikely to change the conclusions of that paper. Most studies have found lower CEA is associated with pCR and GR, either as a continuous or categorical variable with the most common cut-off being 5ng/mL.

Table 1.6. CEA as a predictor of pathological response to nCRT for rectal cancer

CEA classification	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
Continuous variable (ng/mL)	885	pCR	OR 1.03 (1.01-1.06), p=0.03	Y	Retrospective	(37)
Continuous variable (log2 ng/mL)	620	pCR	OR 0.861 (0.743-0.999), p=0.049	Y	Retrospective	(48)
Continuous variable (ng/mL)	947	pCR	OR 2.39 (1.53-3.74), p<0.001	Y	Retrospective	(75)
Continuous variable (ng/mL)	64	pCR; Good response (dTRG)	5.62 vs. 22.27 ng/mL, p=0.002; 6.30 vs. 27.86 ng/mL, p<0.001	N	Prospective	(76)
Continuous variable (ng/mL)	148	dTRG	NS	Y	Retrospective	(69)
Continuous variable (ng/mL)	73	pCR	NS	-	Retrospective	(77)
Elevated	18,113	pCR	OR 0.65 (0.52–0.77), p<0.001	Y	Retrospective	(34)
Elevated	23,747	pCR	NS	Y	Retrospective	(53)
≤2.5 ng/ml	148	pCR	Associated with higher pCR rate	N	Retrospective	(78)
<2.5/ ng/mL / ≥2.5 ng/mL	242	pCR	NS	Y	Retrospective	(38)
≤3/3–6/ 6–9 ≥9	352	Good response (dTRG)	≤3 = reference, p<0.001 3-6: OR 0.34 (1.124-0.919) for 6-9: OR 0.15 (0.058-0.401)	Y	Retrospective	(79)

CEA classification	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
≤5ng/mL	274	pCR	Predicted pCR if <5ng/mL, no OR given	Y	Retrospective	(60)
>5ng/mL	469	pCR	OR 0.90 (0.81–1.00), p=0.04	Y	Retrospective	(40)
≤5ng/mL	609	dTRG	OR 2.53 (1.63-3.92), p<0.001	Y	Retrospective	(70)
≤5ng/mL	212	pCR	NS	-	Retrospective	(80)
<5ng/mL	86	dTRG	NS	Y	Prospective	(45)
<5ng/mL	391	pCR	OR 2.66 (1.38-5.12), p=0.004	Y	Retrospective	(51)
<5ng/mL	351	pCR	OR 3.08 (1.29-7.37), p=0.011	Y	Retrospective	(81)
<5ng/mL	260	pCR	OR 9.32 (2.16–40.19), p=0.03	Y	Retrospective	(67)
<5ng/mL	168	pCR	NS	Y	Retrospective	(41)
≤10ng/mL	12	dTRG	NS	Y	Retrospective	(39)

Dreyer et al investigated the validated SIRS marker modified Glasgow Prognostic Score (mGPS) which is calculated by assigning a score of one each for an elevated C-reactive protein and hypoalbuminaemia and found mGPS pre-CRT was a strong independent predictor of GR on logistic regression (44).

1.2.3.2.2 Haematological Predictors

In two of six studies increasing haemoglobin was associated with pCR. In both studies it was assessed as a continuous variable without a defined cut-off. A real difference is possible but the clinical significance of this is not likely to be large. Circulating lymphocyte ratio (82) and T helper and cytotoxic T cells (47) have been associated with GR in small retrospective studies. Fibrinogen levels were associated with pCR [OR 2.026 (1.369-2.997), $p<0.001$] in a single study of 947 patients (75). Multiple studies have examined the neutrophil to lymphocyte ratio and none found it predictive of PR (44, 77, 82-85). Sun et al found no association between circulating tumour cell numbers and GR in 115 patients (86).

Table 1.7. Haematological parameters as predictors of pathological response to nCRT for rectal cancer

Predictor	n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
Haemoglobin						
Continuous variable	573	pCR	OR 1.30 (1.10–1.53), p=0.002	Y	Retrospective	(48)
Continuous variable; anaemia	173	pCR	OR 1.04 (1.00–1.07), p=0.05; anaemia NS	Y	Retrospective	(83)
Continuous variable	73	pCR	NS	-	Retrospective	(77)
Continuous variable	885	pCR	NS	Y	Retrospective	(37)
Continuous variable / Hb < 12 g/dl or ≥ 12 g/dl	464	pCR	NS	Y	Retrospective	(87)
Normal/ Anaemia	79	dTRG	NS	-	Retrospective	(44)
Circulating lymphocytes	85	pCR; dTRG	pCR NS; lymphocyte ratio ≤24.6% OR for good response 3.99 (1.37-8.28), p<0.01	Y	Retrospective	(82)
	45	dTRG	Th and Tc independent predictors of good response HR = 10.56 (1.5-99.6) and 11.38 (1.5-146.4, p=0.0194) respectively, p=0.0124	Y	Prospective	(47)
Red blood cells	173	pCR	NS	Y	Retrospective	(83)
Platelets	173	pCR	NS	Y	Retrospective	(83)
	314	pCR	<370,0000/uL predictor pCR OR 5.483 (1.27–23.65), p=0.023	Y	Retrospective	(81)

Th = T helper cell; **Tc** = cytotoxic T cells

There is some evidence of a difference between sub-types of TILs in responders and non-responders but these are small studies often lacking multivariate analysis (Table 1.8).

Table 1.8. Tumour-infiltrating lymphocytes as predictors of response to nCRT for rectal cancer

n	Outcome measure	Findings OR (95% CI), p value	Multivariate analysis	Study Design	Reference
31	dTRG	Low density CD8+ TILs associated with poor response	N	Retrospective	(88)
106	TRG	NS	-	Retrospective	(89)
128	pCR; TRG	OR 5.27 (1.62-17.16), p=0.0058 for pCR with low Foxp3 expression; association with TRG on univariate analysis only.	Y	Retrospective	(90)
62	dTRG	Good responders had higher CD81TILs and CD41TILs, and lower MDSC-TILs (high vs low CD81TILs 61.2% vs 32.2%; p=0.022; high vs low CD41TILs 61.2% vs 32.2%; p=0.022; high vs low MDSC-TILs 29.0% vs 64.5%; p=0.005)	N	Retrospective	(91)

1.2.3.2.3 Protein Expression

A large number of protein biomarkers have been evaluated as predictors of response to radiotherapy for rectal cancer. Due to the vast amount of material in the literature, those that have been assessed in more than one study are presented in this section and Table 1.9, and those assessed only in a single study are listed in the appendix (Table A1). Although other methods are available (either antibody or spectrometry based), generally protein expression is assessed using immunohistochemistry (IHC) with antibody-based detection of the protein of interest on a formalin-fixed paraffin-embedded (FFPE) slide, often retrospectively. Any one of a number of scoring systems may be used to semi-quantitatively analyse IHC (92). The advantages of IHC include being able to use historical diagnostic FFPE slides without a need to prospectively obtain tissue and process in a specific manner. It is a technique used routinely in pathological practice and therefore holds potential for easy translation to clinical use. It can also be done with relative efficiency including automated staining allowing relatively large numbers of samples to be processed compared with manual methods such as gel electrophoresis and immunoblotting. It is usually relatively sensitive and specific although this is antibody dependent. Disadvantages include variability in interpretation and poor reproducibility.

Table 1.9. Protein expression assessed using immunohistocheistry as predictors of pathological response to nCRT for rectal cancer

Predictor	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
Ki67	81	pCR	High expression OR 4.48 (1.43-14.09), p=0.01	Y	Retrospective	(73)
	60	Good response (dTRG)	OR 1.17 (1.06-1.29), p=0.002	Y	Retrospective	(93)
	55	pCR; dTRG	NS; Good responders lower expression (70.7% vs. 28.5%, p<0.001)	N	Retrospective	(94)
	46	pCR	NS	Y	Retrospective	(95)
	40	dTRG	NS	-	Retrospective	(64)
	130	dTRG	NS	Y	Retrospective	(96)
	70	TRG	NS	-	Retrospective	(97)
	86	TRG	NS	-	Prospective	(98)
	37	TRG	NS	-	Retrospective	(99)
	112	TRG	NS	Y	Retrospective	(100)
Bax	60	Good response (dTRG)	OR 18.1 (3.11-105.7), p=0.001	Y	Retrospective	(93)
	152	pCR; dTRG	NS	-	Retrospective	(101)
		pCR	NS	-	Retrospective	(73)
Bcl-2	130	dTRG	Correlated with tumour regression	Y	Retrospective	(96)
	152	pCR; dTRG	NS	-	Retrospective	(101)
	86	TRG	NS	-	Prospective	(98)
		pCR	NS	-	Retrospective	(73)

Predictor	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
COX-2	82	pCR	Low Cox-2 expression gave a HR of pCR of 0.205 (0.059-0.708), p=0.012	Y	Retrospective	(102)
	55	pCR; dTRG	NS; Good responder's lower expression (72.9% vs. 22.8%, p<0.001)	N	Retrospective	(94)
	152	pCR; dTRG	NS; high Cox-2 expression correlated with poor response	N	Retrospective	(101)
	30	TRG	NS	Y	Prospective	(103)
	130	dTRG	NS	y	Retrospective	(96)
		pCR	NS	-	Retrospective	(73)
p21	112	TRG	high expression associated with poor pathologic responses OR of good response 0.127 (0.022-0.729), p=0.021	Y	Retrospective	(100)
	70	TRG	NS	-	Retrospective	(97)
	152	pCR; dTRG	NS	-	Retrospective	(101)
	81	pCR	High expression OR 4.65 (1.50-14.46), p=0.008	Y	Retrospective	(73)
p53	70	TRG	NS	-	Retrospective	(97)
	130	dTRG	NS	Y	Retrospective	(96)
	37	TRG	NS	-	Retrospective	(99)
	152	pCR; dTRG	NS	-	Retrospective	(101)
	121	dTRG	P53 type on IHC was positively correlated with response (p=0.039)	Y	Retrospective	(39)
	81	pCR	NS	Y	Retrospective	(73)
EGFR	46	pCR	Low EGFR expression OR 12.9 (1.44-115.5), p=0.007	Y	Retrospective	(95)

Predictor	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
		pCR	NS	-	Retrospective	(73)
VEGF	152	pCR; dTRG	NS; High VEGF expression correlated with poor response		Retrospective	(101)
	46	pCR	NS	Y	Retrospective	(95)
	55	pCR	NS	-	Prospective/ Retrospective	(104)
	85	TRG	NS	-	Retrospective	(105)
	81	pCR	High expression OR 4.34 (1.24-15.11), p=0.02	Y	Retrospective	(73)
TS	46	pCR	High TS expression OR 16.7 (1.8-150.9), p=0.002	Y	Retrospective	(95)
	60	Good response (dTRG)	OR 1.83 (1.11-3.03), p=0.019	Y	Retrospective	(93)
		pCR	NS	-	Retrospective	(73)
GLUT1	40	dTRG	NS	-	Retrospective	(64)
	86	TRG	NS	-	Prospective	(98)
	85	TRG	NS	-	Retrospective	(105)
	104	pCR; TRG	pCR rate 27.8% in low expression vs. 4.0% in high expression group, p=0.012	N	Retrospective	(106)
HIF-1α	104	pCR	NS	-	Retrospective	(106)
	86	TRG	NS	-	Prospective	(98)
	85	TRG	NS	-	Retrospective	(105)
	180	Good response (dTRG)	Low expression of HIF-1α associated with good response	N	Retrospective	(107)

Predictor	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
	55	pCR	NS	-	Prospective/ Retrospective	(104)
HER2	130	dTRG	NS	Y	Retrospective	(96)
	119	TRG	NS	-	Retrospective	(108)
APAF-1	152	pCR; Good response (dTRG)	NS; strong correlation between high APAF-1 expression and good response	-	Retrospective	(101)
	82	pCR	low expression HR 4.29 (1.34-13.70), p=0.014	Y	Retrospective	(102)
Survivin	36	dTRG	NS	-	Retrospective	(109)
	43	Good response (dTRG)	8/26 response in high expression vs. 11/17 in low expression group, p=0.01	N	Retrospective	(110)
	37	TRG	NS	-	Retrospective	(99)

Nine studies have assessed Ki67 protein staining by IHC with outcomes of pCR and TRG; three studies found a difference in expression but they were not all concordant, two found higher expression of Ki67 protein associated with better response (73, 93) while the third found lower expression associated with GR (94).

Bax and Bcl-2 are markers of apoptosis. Kikuchi et al found better TRG was associated with higher expression of Bax on multiple logistic regression in 60 patients (93), but two other studies found no difference (73, 101). One of four studies found positive Bcl-2 staining correlated with poorer dTRG in 130 patients (96). APAF-1 and survivin are also regulators of apoptosis. Two studies of APAF-1 found associations between high APAF-1 expression and GR (101), and high APAF-1 expression and pCR (102). Of three studies investigating survivin two found no difference (99, 109); a third study found better response with lower expression but only included 43 patients (110).

COX-2 is an isozyme of cyclooxygenase (COX), an enzyme responsible for synthesis of prostaglandins and thromboxane from arachidonic acid. COX inhibitors may prevent or slow the development of colorectal neoplasia (111). Three studies found lower expression associated with GR and three studies found no difference. Although these are small studies, any true directional association is unlikely to be strong.

p21 is a cell cycle regulator. Sim et al found high expression of p21 was associated with poorer TRG (100), while Hur et al found higher p21 expression had increased pCR rates compared with low expression (73). Two studies found no difference and overall it can be said no evidence of a directional effect exists. The p53 tumour suppressor gene protein product was examined in six studies, with five finding no significant difference although one study found p53 wild-type was associated with GR when compared to mutant p53 (39).

Epidermal growth factor receptor (EGFR) is a key molecule involved in the MAPK/ERK pathway of colorectal carcinogenesis, and is the target of monoclonal antibody therapy with clinical utility in metastatic colorectal cancer. Low EGFR expression was associated with pCR in one study, another found no difference. Vascular endothelial growth factor (VEGF) has a known role in angiogenesis which is required for tumour growth. No association between VEGF expression and pathological response was found in three studies; high expression was associated with poorer TRG in a single study (101).

GLUT1 and HIF-1α are markers of cellular hypoxia. Multiple studies showed no significant difference for TRG or pCR for either marker (64, 98, 104-106), with one small study showing an association with each marker on univariate analysis only (106, 107).

1.2.3.2.4 Gene Expression Profiling

Gene expression involves DNA sequencing using methods such as real time quantitative polymerase chain reaction. It can be performed on tumour or germline tissue. Predictors based on gene expression profiling are summarised in Table 1.10.

Table 1.10. Gene expression profiling as predictors of pathological response to neoadjuvant chemoradiotherapy for rectal cancer

Predictor	Tissue sequenced	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
KRAS	Tumour	146	TRG	NS	-	Retrospective	(112)
	Tumour	132	pCR	KRAS mutations more common in non-pCR (49% versus 24%, p=0.0145)	N	Prospective	(113)
	Tumour	96	pCR; TRG	NS	-	Prospective	(114)
CCND1	Tumour	132	pCR	Mutations more common in non-pCR	N	Prospective	(114)
MTHFR	Tumour	132	pCR	Mutations more common in non-pCR	N	Prospective	(113)
P53	Tumour	96	pCR; TRG	NS	-	Prospective	(114)
EGFR	Tumour	46	pCR	Low EGFR associated with pCR (p=0.007)	Y	Prospective	(95)
EGFR	Tumour	146	TRG	NS	-	Retrospective	(112)
EGFR	Tumour	40	dTRG	NS	Y	Retrospective	(115)
EGFR	Germline	93	dTRG	NS	-	-	(116).
VEGF	Tumour	40	dTRG	Low VEGF associated with good response. Hr 30.25 (1.72-529.68) P=0.019	Y	Retrospective	(115)
HIF1	Tumour	40	dTRG	NS	Y	Retrospective	(115)
Clock, Cry2 and Per2 (circadian clock genes)	Tumour	40	pCR	Expression of all significantly higher in pCR (p<0.05)	N	Retrospective	(117)
PI3K pathway	Tumour	201	pCR	Wild-type pathway OR 5.146 (1.17–22.58), p=0.030 c.f. mutated pathway	N	Retrospective	(43)

Predictor	Tissue sequenced	n	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
MAPK pathway (RAS, BRAF, MEK)	Tumour	201	pCR	NS	N	Retrospective	(43)
RTK genes	Tumour	201	pCR	NS	N	Retrospective	(43)
XRCC1	Germline	93	dTRG	Significant difference in polymorphisms for good and poor responders	-	-	(116)
ERCC1	Germline	93	dTRG	NS	-	-	(116)
MTHFR	Germline	93	dTRG	NS	-	-	(116)
DPYD	Germline	93	dTRG	NS	-	-	(116)

1.2.3.2.5 Thymidylate Synthase

Thymidylate synthase (TS) is a key enzyme necessary for DNA synthesis, and is inhibited by 5-FU. Alterations in the TS gene (germline or tumour) therefore could influence outcomes for patients by altering natural progression or response to 5-FU treatment commonly used in nCRT.

Carlomagno et al found high TS expression on IHC associated with pCR (95), as did Kikuchi et al (93) but a third study found no difference. Hur et al found no significant difference in tumour regression for TS gene polymorphisms on tumour biopsy in 44 patients with rectal cancer (118). With regard to germline TS polymorphisms, a study of 50 patients found that having at least one TS 3G allele were more likely to have a complete or partial pathological response to 5-FU [OR 10.4 (1.3–81.6), p=0.01] (119). Paez et al also looked at germline TS polymorphisms for 51 patients over 9 years and found on logistic regression analysis only one genotype correlated with PR (120). Germline polymorphisms again examined by Paez et al found no relationship between the different TS genotypes and pCR in 128 patients (121). Tan et al examined germline TS polymorphisms in 135 patients and found no difference between pCR for different genotypes (122). Lamas et al found that TS polymorphisms differed between patients obtaining good and poor responses (116).

1.2.3.2.6 Single Nucleotide Polymorphisms (SNPs)

An SNP involves alteration of a single nucleotide at a specific position in a gene. Such minor alterations can have significant effects on gene expression and function. Numerous SNPs have been investigated in predicting response to CRT for rectal cancer; the affected genes containing SNPs with suggested predictive ability are presented in Table 1.11. Exact polymorphisms can be found in the references.

Table 1.11. Single nucleotide polymorphisms as predictors of pathological response to nCRT for rectal cancer

Gene with SNP	n	Tissue sequenced	Outcome measure	Findings (OR, 95% CI), p value	Multivariate analysis	Study Design	Reference
XRCC1	113	Germline	pCR	OR for non-pCR 3.24 (1.20–8.74), p=0.020	Y	Retrospective	(123)
MSH6	113	Germline	pCR	OR for non-pCR 0.12 (0.03–0.51), p=0.004	Y	Retrospective	(123)
VEGF-α	113	Germline	pCR	OR for non-pCR 3.14 (1.18–8.38), p=0.022	Y	Retrospective	(123)
DROSHA	265	Germline	pCR	OR for non-pCR 1.87 (1.10-3.17), p=0.0207	Y	Retrospective	(123)
TRBP	265	Germline	pCR	OR 0.39 (0.19-0.79), p=0.0089	Y	Retrospective	(123)
SMAD3 (three SNPs)	265	Germline	pCR	OR 2.01 (1.22-3.31), p=0.0064; OR 0.45 (0.24-0.85), p=0.0135; OR 0.48 (0.25-0.94), p=0.0316	Y	Retrospective	(123)
SOD2	71	Germline	dTRG	OR 0.19 (0.06-0.64), p=0.005	Y	Prospective	(124)
IL13	71	Germline	dTRG	OR 0.14 (0.04-0.49), p=0.0008	Y	Prospective	(124)
IL13	46	Tumour	dTRG	NS	-	Retrospective	(125)
CORO2A (two SNPs)	113	Germline	dTRG	OR 0.377 (0.18-0.789), p=0.01; OR 0.205 (0.044-0.944), p=0.03	N	Prospective	(126)
AREG	84	Germline	pCR	OR 0.26 (0.06–0.79), p=0.0149	Y	Retrospective	(127)
ERCC1 (two SNPs)	84	Germline	pCR	OR 0.24 (0.05, 0.73), p = 0.0096; OR not estimable p=0.0238	Y	Retrospective	(127)

1.2.3.2.7 Microarrays and Gene Signatures

Microarrays allow a large number of genes to be sequenced at one time, consisting of a large number of probes attached to a solid surface with subsequent quantification of the abundance of specific nucleic acid sequences targeted. Such an approach has experienced some success in predicting response to chemotherapy for breast cancer (128). Several attempts to use microarrays to predict response to radiotherapy for rectal cancer have been attempted with very limited success (129). Rimkus et al generated gene expression profiles from 43 tumour biopsies and identified a 42 gene expression signature but the positive predictive value for those identified as responders was only 71% (130). Gantt et al identified a 183-gene signature and an 812 gene signature for 33 patients (131). The 812 gene signature had 100% sensitivity and specificity for non-responders while the 183 gene signature had 33% sensitivity and 100% specificity, but the limited generalisability of complex genetic profiling of small numbers of patients is unlikely to yield a clinically useful predictor with generalisability.

1.2.3.2.8 Messenger RNA

Gene expression is dynamic. Biological switches act to regulate gene expression according to biological need. When required, messenger RNA (mRNA) conveys genetic information from the nucleus to the ribosome to determine the amino acid sequence required for protein synthesis. Because cellular processes are complex, mRNA identified does not necessarily result in a protein being produced in meaningful amounts, and therefore it is different to detecting protein by IHC or other methods. mRNA molecules can be identified in tissue or serum, and numerous mRNAs have been explored as predictors of response to radiotherapy for rectal cancer. Each mRNA is likely to have a biological function specific to the protein it codes but the function of many mRNA molecules are not yet known. mRNA is generally sequenced using quantitative reverse transcription polymerase chain reaction.

Huh et al looked at 13 mRNA biomarkers in 123 tumour biopsies and found elevated expression of CD44 was predictive of poorer TRG [OR 4.694 (1.155-17.741), p=0.030]; there was no difference for pCR (132). They found no difference for p53 or CD133, among others (132) while in contrast, Hur et al found mRNA expression for p53, p21, Ki67, and CD133 was significantly associated with GR and pCR, but there was no difference for CD44 (133). In both

this study and their 2014 study (73) they attempted to create a biomarker based scoring system with limited success (133).

Decreased neuronal pentraxin 2 mRNA in 40 patients was associated with better TRG and pCR. Mean CXCL10 mRNA expression was significantly higher in non-PCR patients but there was no difference in CXCL10 protein expression by IHC in the same patients (59), making it less likely to be a truly significant biological effect.

1.2.3.2.9 Micro-RNA

Micro-RNA (miRNA) are small noncoding regions of RNA that have a role in regulation of gene expression (134). miRNA with suggested predictive potential are summarised in Table 1.12. As with many novel biomarker studies, these are best considered pilot studies as they often include small numbers of patients and there is no clinical application at the current time. Mi-RNA are generally sequenced using quantitative reverse transcription polymerase chain reaction.

Table 1.12. Micro-RNA predictors suggested to have potential as predictors of pathological response to nCRT for rectal cancer

miRNA	n	Source tissue	Outcome	Findings (OR, 95% CI), p value	Multivariate analysis	Reference
miR-21	92	Tumour	pCR	High expression OR 9.75 (2.24 to 42), p=0.01	Y	Carames, Cristobal (135)
miR-31	82	Tumour	dTRG	Overexpression predicted poor response OR 0.18 (0.06 to 0.57), p=0.003	Y	(136)
miR-125b	38 tumour; 34 serum	Tumour and serum	dTRG	High expression associated with poor response	N	(137)
miR-194	38	Tumour	dTRG	Significantly upregulated in responders	N	(138)
miR-145	40	Tumour	dTRG	Major response correlated with higher expression (p=0.031)	N	(139)
Set of 13 miRNA	38	Tumour	pCR	Strongly associated with pCR	N	(140)
miRNA-345	129 serum (training and validation) and 20 tumour	Tumour and serum	dTRG	High expression correlated with poor response in tumour. miR-345 expression significantly downregulated in CRT-sensitive group (p=0.007)	N	(141)

1.2.3.2.10 Gene Methylation

Methylation is a way of regulating gene expression according to biological need. Methylation of genes associated with radiosensitivity could therefore play a role in predicting response to nCRT. Ha et al explored the association of a selection of such genes with TRG and suggested methylation of KLHL34 may be predictive but the finding was of borderline statistical significance (142). Molinari et al examined methylation across a large number of genes in 74 rectal cancer biopsies and found TIMP3 methylation was statistically different across four TRG classes on ANOVA, but the main difference appeared to be between Dworak TRG 1 (minimal regression) and 2 (moderate regression) which is of limited clinical benefit (143). Tsang et al found global methylation in 53 rectal cancer biopsies correlated with a modified 3-point TRG ($p<0.001$), with a significant difference between pCR and partial PR (144).

1.2.3.2.11 Chromosomal Alterations

A cytogenetics approach found tumour cell chromosomal alterations in 1p, 1q, 11p, 12p and 17p were associated with grouped TRG in 45 patients (145). Higher rates of error in chromosome segregation enhanced pathological response in 62 patients [OR 3.9 (1.18-12.91), p=0.02], and when combined with decreased levels of the DNA damage repair protein Mre11 portended a markedly enhanced response (OR 54.0, 95% CI not provided, p=0.008) (146). A cytogenetics microarray approach called 'array Comparative Genomic Hybridization' analysis of tumour biopsies in 48 patients found differences between a large number chromosomal alterations in responders vs. non-responders to CRT based on dTRG although the clinical application of this, like specific gene signatures of complex molecular panels, is not immediately realisable (147).

1.2.3.3 Radiological Predictors

Advanced imaging characteristics are considered in this category. These include any radiological parameters over and above routine clinical staging. Radiological modalities investigated in the period of this systematic review included computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). CT texture analysis was found not to be a predictor of GR in 95 patients (148).

1.2.3.3.1 Magnetic Resonance Imaging (MRI)

Table 1.13. MRI parameters as predictors of pathological response to nCRT for rectal cancer

Parameters assessed	n	Outcome	Findings	Multivariate analysis	Study Design	Reference
ADC (dwMRI)	100 (n=50 pCR, n=50 non-pCR)	pCR	ADC poor predictor with AUC of ROC 0.670	N	Retrospective	(149)
ADC (dwMRI)	76	pCR	NS	-	Retrospective	(150)
ADC (dwMRI)	64	pCR	NS	-	Prospective	(151)
ADC (dwMRI)	34	dTRG	NS	-	Prospective	(152)
ADC, tumour volume (dwMRI)	59	pCR	ADC lower in pCR group (p=0.010). AUC of ROC was 0.77 for predicting pCR	N	Prospective	(153)
ADC histogram analysis (dwMRI)	86	pCR	NS	N	Retrospective	(154)
ADC, pseudodiffusion (D*) coefficient, true diffusion coefficient (D), perfusion-related fraction (f). (T2/dwMRI)	98	pCR	NS	-	Prospective	(155)
D_{app}, K_{app} and ADC (T1/T2/dwMRI/DCE MRI)	41	dTRG	$D_{app\text{-}10th}$ lower in good responders than poor responders with AUC 0.752 (p 0.036); ADC NS	N	Retrospective	(35)

Parameters assessed	n	Outcome	Findings	Multivariate analysis	Study Design	Reference
K_{trans}, K_{ep} and v_e (DCE MRI)	38	pCR	Differences for three parameters between pCR and non-pCR ($p<0.05$). ROC AUC for K_{trans} 0.92 (0.84, 1.00); K_{ep} 0.67 (0.47, 0.87); v_e 0.66 (0.46, 0.87)	N	Prospective	(156)
Perfusion parameters (DCE MRI)	30	dTRG	Late slope differed between good and poor responders, AUC 0.90	N	Prospective	(157)
PI_{mean} (DCE MRI)	83	pCR	NS	Y	Prospective	(158)
Fractal-based radiomics (T2 MRI)	198 (n=173 training set, n=25 validation set)	pCR	Predictive model using cT, cN, skewness, entropy and max fractal dimension (FD) with AUC 0.77 +/- 0.07 (training) and AUC = 0.79 ± 0.09 (validation set)	Y	Retrospective training set, prospective validation set	(159)
Tumour compactness (T2 MRI)	122	pCR	HR 4.103 (1.801-9.346), p=0.001.	Y	Retrospective	(71)
T2 signal intensity and tumour volume (T2 MRI)	48	pCR; dTRG	NS	-	Prospective	(160)
Intensity histogram analysis (T2 MRI)	162 teaching, 59 validation sets	pCR	Logistic regression model using covariates cT, skewness and entropy. Model AUCs were 0.73 (internal) and 0.75 (external).	Y	Retrospective	(161)
EMD for T3 invasion (T2 MRI)	111	pCR; dTRG	EMD significantly higher in non-pCR (7.8 ± 3.2 mm) than pCR (6.1 ± 1.8 mm), p=0.033; NS for dTRG.	N	Retrospective	(162)

Parameters assessed	n	Outcome	Findings	Multivariate analysis	Study Design	Reference
EMD for T3 invasion (T2 MRI)	118	pCR	Less T3 invasion associated with pCR [OR, 0.74 (0.62–0.90), p<0.002]. Likelihood of pCR decreased by 35% for every mm of EMD invasion.	N	Retrospective	(163)

ADC = apparent diffusion co-efficient; **EMD** = extramural depth of invasion; **PI** = perfusion index; $\mathbf{D_{app}}$ = apparent diffusion parameter of Gaussian distribution; $\mathbf{K_{app}}$ = apparent kurtosis coefficient; **DCE** = dynamic contrast enhanced; $\mathbf{v_e}$ = extracellular extravascular space volume fraction; $\mathbf{K_{trans}}$ = volume transfer constant; $\mathbf{K_{ep}}$ = rate constant (K_{trans}/v_e).

MRI is an imaging modality with a large number of techniques possible, each with different uses. Diffusion weighted MRI allows mapping of water molecules as they move in biological tissues, and has received the most attention of MRI techniques in attempts to predict response to nCRT. Of the eight studies assessing perfusion parameters none have provided strong evidence to support this modality as a predictor.

Two studies found less extra-mural invasion of T3 tumours was associated with greater chance of pCR but these studies did not perform multivariate analysis (162, 163). The clinical utility of this is doubtful in the context of only modest predictive ability of clinical T stage overall; to sub-classify T3 into responders and non-responders seems unlikely to allow advancement in patient selection for radiotherapy.

Dynamic contrast enhanced (DCE) MRI measures T1 changes in tissues over time after gadolinium contrast is administered intravenously and allows assessment of perfusion parameters. Martens et al found the late slope had an excellent AUC in their small 30 patient study (157) but this requires further investigation.

Radiomics is a term used to describe the conversion of radiological images into data that can then be examined. Cusumano et al used this approach and developed a model that was internally validated (159), but this approach still requires external validation.

1.2.3.3.2 Positron Emission Tomography (PET)

PET holds interest as a predictor because it is a type of functional imaging demonstrating metabolic activity which may be associated with treatment response. Although structural elements may contribute to response e.g. T and N stage, functional imaging usually aims to combine structural and functional information. Fluorodeoxyglucose (FDG) PET is based on the premise that tumours consume large amounts of glucose as a preferential energy substrate, even in the presence of normally functioning mitochondria, a phenomenon known as the Warburg Effect (164). FDG is the most common tracer and was used in all studies except Withofs et al (165).

Numerous studies have explored PET, usually combined with CT, as a method to predict response. The results are presented in Table 1.14, and while there have been differences seen in parameters between good responders and poor responders, no study has resulted in a predictor with adequate utility for clinical use.

Table 1.14. PET parameters as predictors of pathological response to nCRT for rectal cancer

Parameters assessed	n	Outcome	Findings	Multivariate analysis	Study Design	Reference
SUV, MTV, TLG, textural analysis	74	dTRG	MTV calculated using SUV_{mean} of liver associated with TRG; others NS	Y	Retrospective	(36)
SUV_{max}, MTV	35	dTRG	NS	-	Retrospective	(166)
SUV	31	dTRG	NS	-	Prospective	(167)
SUV_{max}, SUV_{mean}, MTV and TLG	103	pCR	NS	-	Retrospective	(168)
SUV_{max}, MTV, TLG	81	pCR	NS	-	Retrospective	(169)
SUV_{max}, $SUV_{mean,}$ MTV, TLG	64	pCR; dTRG	MTV-pre lower in pCR group (9.87 vs. 14.62 cm3, p = 0.045)	N	Prospective	(76)
SUV_{max}, MTV, TLG, others	69	dTRG	NS	-	Retrospective	(170)
SUV_{max}	80	TRG	Median SUV_{max} at baseline higher for TRG 1 (minimal response) compared other TRG categories	N	Prospective	(171)
SUV_{max}	99	pCR	NS	Y	Prospective	(172)
[18F]$FPRGD_2$ SUV_{max}, SUV_{mean}	32	pCR; TRG	[18F]FPRGD2 uptake higher in pCR; SUV_{mean} moderately correlated with TRG (Spearman's r= −0.41, p=0.019).	N	Prospective	(165)

SUV = standardised uptake value; **MTV**= metabolic tumour volume; **TLG** = total lesion glycolysis

1.2.4 Discussion

This systematic review examines the last ten years of literature investigating predictors of pathological response to neoadjuvant chemoradiotherapy for rectal cancer. It provides a broad overview of a large topic, attempting to provide context and background for the large number of predictors identified and the different techniques employed in attempts to predict response.

Of the large amount of literature examined, several predictors appear to have current clinical utility. Clinical predictors including advanced T and N stage, advanced tumour size and mucinous histology have moderate evidence to support their roles as predictors of poor pathological response. It is important to consider that the majority of these factors are also relative indications for neoadjuvant radiotherapy; although the response is less likely to be good, a good response is also of greater clinical importance. The only clinically useful biomarker available is CEA, and an elevated CEA has been found predictive of poor pathological response also. The role of advanced imaging characteristics is evolving, but currently there is not adequate evidence to support advanced imaging as a predictor in clinical practice. As individual predictors, none are strong enough to allow treatment decisions to be based on them currently.

There are numerous limitations of a systematic review of this nature. The large amount of heterogeneity in the literature prevents quantitative assessment by meta-analysis, although this is possible for selected predictors. This review considers any pathological measure of regression, including pCR or a recognised TRG. This has the benefit of identifying a large number of additional predictors that have not been reported for pCR, but brings much more heterogeneity to the subject which makes interpretation difficult. There are multiple different TRG systems, and no attempt has been made to relate the specific TRG grading system used in each instance, although they have some common features and are dichotomised in a similar way to categorise patients as good responders or poor responders. Although not formally assessed, it is likely there is significant publication bias in the literature reviewed due to the presence of a large number weakly positive studies with low participant numbers.

Study size is important when considering a pCR rate may be <20%, small studies will have very low numbers in a pCR group. This may often be the reason dichotomised TRG is used as

an outcome, although it provides different information to pCR. This review limited studies to those with >30 participants, but this could result in five patients or less with pCR, or approximately 15 with GR by dTRG. Many biomarker studies contained small numbers of patients meaning they were really pilot/exploratory studies.

Several studies reported scoring systems using either clinical factors or biomarkers as predictors. Small numbers of participants was common and such results are rarely generalisable to other patient populations.

The most clinically relevant predictors at the current time are clinical T and N stage, and CEA level. These predictors are pieces of routinely available clinical information with reproducibility between observers and no additional cost incurred. Expert radiologists are likely to be concordant when following appropriate reporting guidelines. These predictors have been assessed in the largest number of patients including up to three large retrospective studies with more than 18,000 participants each. Although these predictors offer some utility, this is limited and there is a clear need for better predictors to be identified in order to significantly inform treatment decisions. In the following three chapters an attempt is made to progress this field by investigating retrospective clinical predictors in a local cohort, and then exploring novel biomarker predictors.

2 Clinical Predictors of the Pathological Response to Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer at Christchurch Hospital

2.1 Introduction

There is potential clinical benefit in predicting the response to chemoradiotherapy for patients with rectal cancer. This includes a recommendation of chemoradiotherapy if a good response is likely, either as neoadjuvant treatment or planned non-operative management, or a recommendation to proceed straight to surgery if a poor response is likely. The current standard of care is that patients with locally advanced rectal cancer (LARC) will be offered neoadjuvant radiotherapy if there are not contraindications. Therefore if radiotherapy is indicated they are likely to receive it unless there was a reliable predictor of poor response and up to 40% of patients will experience minimal-poor response (26). Radiotherapy for rectal cancer carries significant morbidity (25, 173), and in the New Zealand setting often requires extended periods of geographical and social isolation for patients and families from rural regions who need to attend a radiotherapy centre hundreds of kilometres away. While pathological complete response (pCR) is a robust outcome measure and has been a major focus of the literature, this is not the only measure of clinical utility. Predicting a good or poor response may also be useful.

Clinicopathological predictors are important as they include information routinely available in the clinical environment. The literature regarding clinical predictors of response to neoadjuvant chemoradiotherapy (nCRT) for rectal cancer has been extensively reviewed in Chapter 1. In summary, factors associated with pCR include smaller tumour size, non-circumferential tumours, lower clinical T and N stage, well-differentiated, non-mucinous and signet ring histological sub-types and lower pre-treatment carcinoembryonic antigen (CEA) level (37, 48, 51, 53, 63, 68, 174). Significant variation has been found globally for the pCR rate to nCRT for rectal cancer. A retrospective study of 297 patients from Auckland, New Zealand examining clinical predictors found a pCR rate of approximately 10% and included historical data now up to 16 years old. Christchurch Hospital is a major tertiary referral centre treating among the greatest volume of rectal cancer patients of any centre nationally, but the pCR rate and predictors of pCR have not previously been investigated.

Clinical scoring tools are common and some have become established in routine medical and surgical practice (175-177). Maximal utilisation of clinicopathological information might be achieved through development of a clinical risk scoring tool to predict the response to nCRT for rectal cancer. Previous attempts to create a predictive model have been of limited predictive ability, are mathematically complex and do not allow easy use in the clinical setting (48, 51), or are from an era before high-quality total mesorectal excision (TME) was routinely performed and did not report on pCR or TRG as an outcome measure (178).

The primary aim of this study was to identify pre-treatment clinicopathological factors that correlate with tumour regression grade (TRG) and/or pCR after nCRT followed by TME for rectal cancer. The secondary aim was to develop a simple clinical scoring system to predict response to radiotherapy for rectal cancer.

2.2 Method

This was a retrospective observational study performed at Christchurch Hospital, a tertiary referral hospital in New Zealand. Ethics approval was obtained from the Southern Health and Disability Ethics Committee (ref: 18/STH/150); individual patient consent was not required. Patients with a histological diagnosis of cancer from a rectal specimen were identified from the Christchurch Hospital Department of Pathology database from August 1 2013 to August 1 2018. The electronic clinical record of all patients identified was examined. The inclusion criteria was adult patients (>18 years of age) with histologically confirmed rectal adenocarcinoma, treated with neoadjuvant long-course radiotherapy and concurrent fluoropyrimidine-based chemotherapy followed by TME. Patients with distant metastatic disease (stage IV disease) diagnosed prior to TME were excluded.

Clinicopathological data was collected from the electronic clinical records including pre-treatment patient and disease factors, treatment and post-treatment variables including the presence of a pCR and the American Joint Committee on Cancer (AJCC) TRG (Table 2.1). All data required has been kept electronically in Christchurch Hospital since prior to the study period. The AJCC TRG is routinely reported for all rectal cancer treated with neoadjuvant radiotherapy in Christchurch Hospital; multiple pathologists report rectal cancer specimens. Data was recorded in a secure Microsoft Excel spreadsheet and analysed using IBM SPSS 25. TRG was dichotomised to categorise patients into two groups, good responders (TRG 0-1) or minimal-poor responders (TRG 2-3). Analysis was performed for the outcome variables of

pCR and good response (TRG 0-1). Univariate analysis was performed using the χ^2 test or Fisher's exact test for categorical variables, the Mann-Whitney U test for nonparametric continuous variables and independent samples t test for parametric continuous variables. Variables which were significant or near significant ($p<0.15$) on univariate analysis were entered into a binary logistic regression model. An attempt to create a clinical scoring tool using a binary score (0 or 1) for significant predictors was attempted, with predictive ability assessed using the area under the curve (AUC) of the receiver operating curve (ROC).

Table 2.1. AJCC tumour regression grading system (reproduced from reference (179))

Description	TRG
No viable cancer cells (complete response)	0
Single cells or small groups of cancer cells (moderate response)	1
Residual cancer outgrown by fibrosis (minimal response)	2
Minimal or no tumour kill; extensive residual cancer (poor response)	3

Neoadjuvant treatment for rectal cancer at Christchurch Hospital routinely includes 45 Gray whole pelvis irradiation and a 5.40 Gray boost to the tumour in 28 fractions over five and a half weeks, with concurrent capecitabine or 5-fluorouracil (5-FU) dose adjusted as clinically indicated.

2.3 Results

Over the five years of the study 470 patients were seen with rectal cancer, of which 51 patients received pre-operative short-course radiotherapy and 195 patients received long-course chemoradiotherapy. Thirty-one (31/195) long-course chemoradiotherapy patients were excluded because they had metastatic disease at presentation or did not undergo TME, leaving a study population of 164 patients. 117/164 (71.3%) were male and the median age of all patients was 66 years (range 31-85 years) (Table 2.2). pCR and TRG rates are shown in Table 2.3. The pCR rate was 14.6% overall, although there was an apparent increase over time with a pCR rate of 19.4% for the last three years of the study. TRG was available for 160/164 patients, therefore analysis for outcome by TRG considers only these patients.

Table 2.2. Demographics and clinical stage of study population

Age (years), median (range)	66 (31-85)	
Gender		
Male	117	71.3%
Female	47	28.7%
Ethnicity		
NZ European	144	87.8%
Maori	6	3.7%
Asian	7	4.3%
Other European	23	14.0%
Other	23	14.0%
Clinical T stage		
T2	27	16.5%
T3	97	59.1%
T4	40	24.4%
Clinical N stage		
N0	32	19.5%
N1	64	39.0%
N2	68	41.5%

Table 2.3. AJCC tumour regression grade (TRG) and pCR rates for all patients

	Frequency	**%**
AJCC TRG		
0	25	15.6
1	45	28.1
2	66	41.3
3	24	15.0
pCR	24*	14.6

**pCR rate not equal to TRG 0 because TRG does not consider LN involvement.*

Table 2.4 compares pre-treatment clinicopathological variables by pCR status and dichotomised TRG. On univariate analysis clinical nodal stage (cN) was lower in the pCR group than non-pCR group, and tumour length on MRI was shorter. Good responders had an older median age by four years (68 vs. 64 years), lower clinical tumour stage (cT) and shorter tumour length on MRI. Maori patients were significantly less likely to get a good response; although only six Maori patients were included none of these patients achieved a good response. This result was not explained by advanced clinical stage at diagnosis. There was also a significant difference for tumour position with good responders more likely to have

anterior tumours (32.4% vs. 12.5%) and less likely to have circumferential tumours (30.9% vs 45.0%).

Table 2.4. Univariate analysis of pre-treatment clinicopathological factors grouped by pathological complete response and good response (TRG 0-1)

	pCR (n=24)	**non-pCR (n=140)**	***p value***	**Total**	**Good response (TRG0-1) (n=70)**	**Minimal-poor response (TRG2-3) (n=90)**	***p value***	**Total**
Age (years) median	69.00	65.00	0.085		68.00	64.00	0.048	66.00
Gender			0.300				0.692	
Male	15 (12.8%)	102 (87.2%)		117	51 (44.7%)	63 (55.3%)		114
Female	9 (19.1%)	38 (80.9%)		47	19 (41.3%)	27 (58.7%)		46
Ethnicity[#]								
NZ European	21 (14.6%)	123 (85.4%)	0.961	144	61 (43.3%)	80 (56.7%)	0.735	141
Maori	0 (0.0%)	6 (100%)	0.301	6	0 (0.0%)	6 (100.0%)	0.028	6
Asian	2 (28.6%)	5 (71.4%)	0.286	7	3 (50.0%)	3 (50.0%)	0.753	6
Other European	2 (8.7%)	21 (91.3%	0.385	23	8 (38.1%)	13 (61.9%)	0.575	21
Other	5 (21.7%)	18 (78.3%)	0.298	23	11 (47.8%)	12 (52.2%)	0.670	23
BMI mean	26.7	28.2	0.257		27.0	28.7	0.090	
Diabetes (on medication)								
Yes	1 (5.9%)	16 (94.1%)	0.295	17	4 (26.7%)	11 (73.3%)	0.156	15
No	22 (15.3%)	122 (84.7%)		144	65 (45.8%)	77 (54.2%)		142
Current smoker								
Yes	2 (10%)	18 (90%)	0.517	20	7 (35.0%)	13 (65.0%)	0.370	20
No	22 (15.5%)	120 (84.5%)		142	63 (45.7%)	75 (54.3%)		138
Clinical T stage			0.106				0.040	
T2	6 (22.2%)	21 (77.8%)		27	14 (53.8%)	12 (46.2%)		26
T3	16 (16.5%)	81 (83.5%)		97	46 (51.0%)	50 (49.0%)		96
T4	2 (5.0%)	38 (95.0%)		40	10 (26.3%)	28 (73.7%)		38
Clinical N stage			0.022				0.089	
N0	9 (28.1%)	23 (71.9%)		32	19 (61.3%)	12 (38.7%)		31
N1	10 (15.6%)	54 (84.4%)		64	25 (40.3%)	37(59.7%)		62
N2	5 (7.3%)	63 (92.7%)		68	26 (38.8%)	41 (61.2%)		67
cN positivity (cN1 or cN2)	15 (11.4%)	117 (88.6%)	0.018	132	51 (39.5%)	78 (60.5%)	0.018	129
Distance from anorectal margin on MRI (mm), median (range)	27.50 (0-150)	31.00 (0-132)	0.988	157	34.50 (0-150)	30.00 (0-132)	0.337	153
Differentiation			0.508				0.893	
Well	0 (0.0%)	3 (100.0%)		3	1 (33.3%)	2 (66.7%)		3
Moderate	14 (13.2%)	92 (86.8%)		106	46 (45.1%)	56 (54.9%)		102

	pCR (n=24)	non-pCR (n=140)	*p value*	Total	Good response (TRG0-1) (n=70)	Minimal-poor response (TRG2-3) (n=90)	*p value*	Total
Poor	0 (0.0%)	6 (100.0%)		6	3 (50.0%)	3 (50.0%)		6
Mucinous histology	2 (28.6%)	5 (71.4%)	0.313	7	5 (71.4%)	2 (28.6%)	0.137	7
Signet ring histology	0 (0.00%)	3 (100.0%)	0.459	3	2 (66.7%)	1 (33.3%)	0.428	3
Position			0.521				0.036	
Anterior	7 (21.2%)	26 (78.8%)		33	22 (68.8%)	10 (31.3%)		32
Posterior	6 (18.2%)	27 (81.8%)		33	14 (42.4%)	19 (57.6%)		33
Lateral	3 (15.8%)	16 (84.2%)		19	9 (50.0%)	9 (50.0%)		18
Circumferential	7 (12.1%)	51 (87.9%)		58	21 (36.8%)	36 (63.2%)		57
Not circ, NOS	0 (0.00%)	9 (100.0%)		9	2 (25.0%)	6 (75.0%)		8
Macroscopic ulceration	14 (33.3%)	42 (66.7%)	0.436	56	29 (52.7%)	26 (47.3%)	0.637	55
Tumour length on MRI (cm), mean	4.04	5.09	0.011	161	13.9	18.5	0.003	157
Tumour length on MRI ≥3cm	20 (13.2%)	131 (86.8%)	0.043	151	61 (41.5%)	86(58.5%)	0.020	147
Tumour length on MRI ≥5cm	7 (10.1%)	62 (89.9%)	0.105	69	22 (33.3%)	44 (66.7%)	0.017	66
Tumour fixity	2 (13.3%)	13 (86.7%)	0.502	15/28 had tumour fixity	6 (42.9%)	8 (57.1%)	0.568	14/27 had tumour fixity
Haemoglobin, mean	132.17	132.73	0.838		135.46	130.68	0.110	
Anaemic	8 (16.3%)	41 (83.7%)	0.672	49	19 (39.6%)	29 (60.4%)	0.509	48
Not anaemic	15 (13.8%)	94 (86.2%)		109	48 (45.3%)	58 (54.7%)		106
Neutrophil, mean	4.47	5.04	0.226	158	4.7742	5.0644	0.397	154
Lymphocyte, mean	2.07	2.01	0.719	158	2.0509	2.0069	0.715	154
CEA, median (range)	2.65 (0.60-26.70)	3.15 (0.60-248.00)	0.166	154	3.00 (0.60-248.00)	3.15 (0.60-180.00)	0.392	151
CEA <5	18 (18.4%)	80 (81.6%)	0.043	98	45 (46.9%)	51 (53.1%)	0.139	96

**statistically significant difference found (p<0.05), [#]multiple ethnicities can be recorded per patient*

When participants with a poor response (TRG 3) were compared with the remaining 136 patients, there was no significant difference in any of the pre-treatment variables examined. Although histological differentiation was different between the groups (χ^2 7.380, 2 d.f., p=0.025) the chi-square test was invalid due to low expected cell counts. Six patients had poorly differentiated tumours identified on pre-treatment biopsy and 2/6 (33.3%) had a TRG 3 compared with 15% of all patients.

2.3.1 Binary Logistic Regression

Binary logistic regression was performed using a stepwise backwards elimination approach for the outcomes of pCR and good response. For pCR the model was initiated with age, cT, cN and tumour length on MRI and CEA as predictors but individual factors only achieved significance when the model was reduced to include only length on MRI, cN stage and age. The χ^2 was 14.779 with 3 d.f. and p=0.002. The Hosmer and Lemeshow test did not demonstrate poor fit (p=0.632). The R^2 statistic was 0.154. Greater length of tumour on MRI and cN2 stage were independent predictors of non-pCR when adjusting for each other and age. cN0 did not reach statistical significance although there was a trend towards increased chance of pCR [OR 2.338 (0.849-6.436), p=0.10].

In the final regression model for predictors of good response, anterior position, circumferential tumours, higher BMI and lower haemoglobin were independent predictors for not achieving good response after adjusting for age, length on MRI and mucinous features on pre-treatment biopsy (Table 2.5). The χ^2 was 31.680, 11 d.f. and p=0.001. The Hosmer and Lemeshow test did not demonstrate poor fit (p=0.457). The R^2 statistic was 0.269.

Table 2.5. Independent predictors of pathological complete response and good response (TRG 0-1) on binary logistic regression

Predictors of pCR	**OR (95% CI)**	***p value***
Length on MRI (cm)	0.676 (0.462-0.990)	0.044
cN2 stage	0.283 (0.080-0.997)	0.050
Predictors of good response (TRG 0-1)	**OR (95% CI)**	***p value***
BMI	0.888(0.815-0.968)	0.007
Anterior position	0.255 (0.068-0.960)	0.043
Circumferential tumours	0.329 (0.108-0.995)	0.049
Haemoglobin	1.028 (1.002-1.054)	0.034

2.3.2 Scoring System

An attempt to develop a clinical risk score for pCR was made by assigning a score of 1 for the events length <5cm, CEA greater than 5 ng/mL and cN0 (all significant on univariate analysis). Scores were generated for 88 participants who had data for all variables, and the scores were 1 (n=16), 2 (n=58) and 3 (n=14). No score significantly improved the AUC of the ROC above 0.5.

Similarly, an attempt to develop a clinical risk score for good response was made by assigning a score of 1 for the events T2-3, N0, anterior tumours and length <5cm (all significant on univariate analysis). This yielded a score for 75 patients (score 0-1=0, score 2=46, score 3=21, score 4=8) who had data for all variables. No score significantly improved the AUC of the ROC above 0.5.

2.3.3 Post-Treatment Variables

Table 2.6 outlines the operative and pathological outcomes for the study population. There was an R0 resection rate of 90.1% and grade 3 TME in 68.8% of patients. Sphincter-preserving surgery (with or without anastomosis), as opposed to abdominoperineal excision, was performed in 65% of cases. On univariate analysis tumours with a higher TRG (poorer response) were significantly more likely to have lymphovascular invasion (p=0.027) and perineural invasion (p=0.007) in the resection specimen (Table 2.7). There was no difference in TRG for an infiltrative tumour margin compared with a pushing margin, when tumour-initiating lymphocytes or a Crohn's-like reaction was present, or for TME grade.

Table 2.6. Operative and pathological outcomes for all patients

	Frequency	%
R0 resection	146/162	90.1
TME grade		
1	18	11.7
2	30	19.5
3	106	68.8
Differentiation		
Well	25	19.2
Moderate	95	73.1
Poor	10	7.7
Mucinous histology	11/141	7.8
Signet ring histology	3/140	2.1
Lymphovascular invasion	22/139	15.8
Perineural invasion	25/139	18.0
ypN		
0	103	62.8
1	42	25.6
2	19	11.6
ypT		
0	27	16.5
1	11	6.7
2	42	25.6
3	68	41.5
4	16	9.8
Sphincter-preserving surgery	104/160	65.0

Table 2.7. Pathological features of resection specimens associated with tumour regression grade on univariate analysis.

AJCC TRG	1	2	3	p value
Lymphovascular invasion	1/44 (2.3%)	15/66 (22.7%)	5/24 (20.8%)	(p=0.027)
Perineural invasion	1/44 (2.3%)	17/66 (25.8%)	7/24 (29.2%)	(p=0.007)

2.4 Discussion

This study describes clinicopathological factors and their association with pathological response, assessed by either pCR or TRG, in 164 patients with stage 1-3 rectal cancer treated with nCRT and TME over a 5 year period at Christchurch Hospital.

The overall pCR rate of 14.6% and pCR rate of almost 20% in the last three years is comparable with published rates worldwide (26). The pCR rate in this study is slightly higher than the 10% previously reported in a New Zealand cohort (68), which may be the most comparable published dataset. Significant regional variation in indications for long and short course radiotherapy are known to exist in New Zealand and may contribute to this difference. Another explanation is the long time period of the previous study (68) which extended back to 2002, and numerous treatment factors may have varied over this time.

For pCR as an outcome measure, this study was largely concordant with the published literature. Clinical N stage was an independent predictor, although a statistically significant difference was only identified for cN2 patients on logistic regression. Decreasing tumour size was associated with pCR in the current study and has been shown by many others (see chapter 1). The literature shows no clear association with distance from the anus and this study found no association with distance from anorectal junction on MRI. Although distance from anal verge on rigid sigmoidoscopy is the gold standard, this was rarely available from the clinical records hence the decision to use the MRI measurement which is included in synoptic reporting. Clinical T stage was only associated with good responders on univariate analysis in this study, and the literature demonstrates cT stage as an independent predictor of pCR only in two very large studies (>18,000 each) suggesting a real but small effect unlikely to be seen in a study this size. Tumour mobility data was very poor in our study with only 28 patients having data available meaning the value of this is minimal. This study had low numbers of patients with mucinous (n=7) and signet ring (n=3) histology, concordant with known percentage of cases of rectal cancer, and it is likely these numbers were too small to identify any association present. Anterior tumour position was found to be an independent predictor of good response (but not pCR) which is a new finding and has not been reported elsewhere, and non-circumferentiality was an independent predictor of good response, concordant with some small retrospective studies reviewed in chapter 1.

It was not possible to identify predictors of poor response (TRG 3), although this remains an important question because there is a relative paucity of published data on this and the clinical utility may be underappreciated. As neoadjuvant radiotherapy is standard of care for LARC, an accurate predictor of poor response could spare a significant proportion of patients from radiotherapy, with major clinical and socioeconomic benefits.

In this study dichotomised TRG was used as an outcome measure and different factors predictive of good response by TRG were identified compared with an outcome of pCR. There are several potential reasons for this. Firstly, a good response is different from a pCR in that not only can small volume disease may remain in the bowel wall, but lymph nodes may also be involved. Expectedly, nodal status is not a predictor of TRG 0 as nodal involvement does not feature in the AJCC TRG. In addition, 43.7% patients were good responders compared with 15% pCR, and the chance of achieving statistical significance was greater with the sample size included.

There have been numerous TRG systems proposed; the AJCC TRG has been in use for over five years at Christchurch Hospital and is routinely available in pathology reports. The Dworak (or a modified version) has been proposed as superior (28) but it was not possible to assess Dworak TRG in this study because it was rarely reported. Research to elucidate the optimal TRG remains important, and would allow some standardisation of research outcomes in the field.

The post-treatment pathological features must be viewed with some caution. Histological differentiation of the resection specimen has not been reported as neoadjuvant treatment can limit interpretability of this, and indeed significant treatment effects can make pathological assessment challenging, including the presence of mucin pools in the absence of mucinous histological sub-type. Lymphovascular and perineural invasion were present more often in those with higher TRG (poorer response) and although this data is not a pre-treatment predictor, LVI and PNI was not identified in any of the diagnostic biopsies therefore no conclusions can be made regarding their presence in pre-treatment tissue. Diagnostic biopsies may be too superficial to identify lymphatic vessels; although lymphatic vessels have been demonstrated in the lamina propria of neoplastic colonic epithelium they don't appear to be present in normal colonic epithelium (180) so the extent of neoplasia will play a role in determining their presence. However, if these features are seen on diagnostic

biopsy this may indicate potential resistance to treatment, and care to identify these features on pathological assessment of diagnostic biopsies may be valuable.

Clinical scoring systems are well-established in medicine and surgery, with wide uptake of scoring systems for predicting severity of pancreatitis (177), pneumonia (175), and mortality associated with sequential organ failure in the intensive care unit (176). For a scoring system to be generalisable it should usually not be mathematically complex, especially if developed on a relatively small study population. Complexity is also likely to decrease uptake in clinical practice. Furthermore, for a scoring system to be useful it must provide additional information over what is immediately apparent on clinical assessment.

Although previous scoring systems have been created to predict response to nCRT for rectal cancer they have not achieved uptake due to a combination of these factors. Joye et al identified a number of clinical variables associated with pCR on logistic regression, and used a ROC to assess the discriminative ability of the prediction models (48). Although they achieved statistical significance the AUC for the ROC was 0.609 proving it an inadequate tool for prediction. Huh et al stratified patients into four risk groups based on clinical predictors identified on logistic regression (51). A group categorised as 'low risk' was most predictive of response to therapy but the predictive ability remained inadequate for clinical use with a sensitivity of 64.1%, specificity of 73.7% and AUC of ROC 0.706.

A major difficulty in creating a scoring system in this setting is a relatively small number of independent predictors, and the fact the effect size of each is not great. A clinical scoring system is unlikely to be of utility in predicting the response to nCRT and other avenues should be explored.

3 The Role of Mitochondria, Oxidative Stress and Antioxidants Including Peroxiredoxins in Radiation Sensitivity for Rectal Cancer: Narrative Literature Review

Previously published and co-authored work.

Fischer J, Eglinton TW, Frizelle FA, Hampton MB. Peroxiredoxins in Colorectal Cancer: Predictive Biomarkers of Radiation Response and Therapeutic Targets to Increase Radiation Sensitivity? Antioxidants (Basel). 2018 Oct 5;7(10). pii: E136. doi: 10.3390/antiox7100136.

3.1 Introduction

Radiotherapy is a vital tool in cancer therapy, and is used in the treatment of a wide range of malignancies including gastrointestinal, genitourinary, head and neck, central nervous system and skin cancer. Radiotherapy may be used as a sole treatment, or in a neoadjuvant or adjuvant setting when combined with surgery. Radiotherapy is used commonly for rectal cancer due to proven benefit in reducing the rate of local recurrence, but the response is highly variable, with approximately 20% of patients experiencing a pathological complete response (pCR), and up to 40% demonstrating minimal regression or even tumour progression (26). The ability to predict the response to radiotherapy could crucially inform the decision when considering radiotherapy for rectal cancer. Patients likely to experience a poor response may be best to proceed straight to surgery thereby avoiding treatment delay and morbidity of radiotherapy; those predicted to have a good response would be best to receive radiotherapy and may even be considered for non-operative management if a complete clinical response is achieved, thereby avoiding the significant risk of mortality and morbidity with rectal cancer surgery.

Ionising radiation (IR) kills cells by directly damage to biomolecules and the generation of reactive oxygen species during the radiolysis of water (181). This immediate damage is not the only challenge faced by irradiated cells. Redox homeostasis can be disrupted for several weeks, compromising the viability of progeny and bystander cells. While the exact mechanisms of redox disruption are unclear, irreparable damage to nuclear and mitochondrial DNA is thought to increase cellular oxidant production and/or compromise antioxidant defences, ultimately leading to sustained oxidative stress and cell death (182).

Cells irradiated in the absence of oxygen are considerably more resistant to IR (183) confirming the importance of oxidative stress. Increased expression of manganese superoxide dismutase (MnSOD) and mitochondria-targeted catalase have both been shown to protect against IR-induced cell death (184-187) and chronic glutathione depletion increases radiosensitivity (188). In this review we focus on the peroxiredoxin family of antioxidant proteins (Figure 3.1). These thiol-dependent peroxidases are abundant in mammalian cells and effectively reduce hydroperoxides (189, 190). Humans have six different peroxiredoxins (Prx1-6) with varying cellular locations: peroxiredoxin 1, 2, and 6 are present in the cytoplasm and nucleus; peroxiredoxin 3 present solely in mitochondria; peroxiredoxin 4 present solely in the endoplasmic reticulum and peroxiredoxin 5 in the cytoplasm, mitochondria, and peroxisomes (191). The catalytic activity of the peroxiredoxins is dependent on an active cysteine (Cys) site that is oxidized to a sulfenic acid by hydroperoxides. For Prxs 1-4 a resolving Cys on the second subunit of the homodimer forms an intermolecular disulfide bond. Conversion back to the reduced state requires thioredoxin or glutaredoxin activity, and in cells under increased oxidative stress, the oxidized forms accumulate. In various systems we have observed that the redox status of endogenous peroxiredoxins can act as a sensitive biomarker of redox homeostasis (192, 193).

All peroxiredoxins have been shown to have altered expression in human cancer (191). In this chapter we review the role peroxiredoxins play in radiation sensitivity for colorectal cancer (CRC), their potential as predictive biomarkers of radiation sensitivity, and to consider the therapeutic implications.

3.2 Radiation Therapy for Colorectal Cancer

Approximately one third of cases of CRC are of rectal origin which affects about one in 60 adults in the Western world (194). While colon and rectal tissue is histologically similar, the clinical behaviour and management of colon and rectal cancer differs significantly and they have also demonstrated different outcomes to adjuvant chemotherapy in a clinical setting (195). Colon cancer is usually treated with bowel resection with or without adjuvant chemotherapy and radiotherapy is rarely used, in contrast with rectal cancer treatment where radiotherapy is common. Historically, local recurrence has been a significant issue following surgery for rectal cancer. The development of improved surgical technique with total mesorectal excision and the use of preoperative radiation therapy has significantly

reduced local recurrence rates (7, 8). In addition, the anatomical arrangement of the rectum in the pelvis away from small bowel allows tumour targeting with less radiation delivery to the vulnerable small bowel. The addition of fluoropyrimidine-based chemotherapy such as 5-fluorouracil to a radiotherapy regimen improves effectiveness of this treatment (13). The highly variable response rate of rectal cancer to radiotherapy is however a major challenge in the management of patients with the disease.

There is a significant shift occurring in the approach to rectal cancer treatment. In selected cases, organ preservation (i.e. omitting resection of the rectum) is offered after neoadjuvant chemoradiotherapy if a complete clinical response (determined by clinical and endoscopic examination, and radiological re-assessment with MRI) is achieved. The omission of surgery has major implications in that the chance for early cure with surgery may be missed, and death from rectal cancer progression may result. The benefits of omitting surgery are a reduction in morbidity and mortality resulting from surgery. For rectal cancer surgery these risks are significant, with an in-hospital mortality rate of 1-2%, over 30% of patients having significant post-operative complications and 20-30% of patients requiring a permanent stoma (4). Similarly, radiotherapy also carries significant morbidity (25, 173), and if a poor response could be reliably predicted then the omission of radiotherapy would be in the patient's best interests and they should proceed directly to surgical resection. While there has been a large volume of research investigating predictors of pCR which can be classified as clinicopathological, radiological and biomarkers, but no robust predictors have been identified (26).

3.3 Redox Homeostasis, Mitochondria and Radiosensitivity

Irradiation of cells causes direct damage to biomolecules and the radiolysis of water. Within fractions of a second, a series of reactive radical species are generated, and in the presence of oxygen this results primarily in superoxide, hydrogen peroxide and hydroxyl radical formation (181), and also reactive nitrogen species (181). As well as this early acute burst a persistent increase in oxidative stress for hours to days after radiation exposure has been reported (181). This sustained stress, if not lethal, is passed to daughter cells, implicating alterations to nuclear or mitochondrial genomes (182).

Mitochondria are prominent sources of reactive oxygen species and targets of oxidative stress, and are hypothesized to be a major target of injury by radiation. In 1965 Goldfeder

first hypothesized mitochondria play a role in radiosensitivity, based on the fact that cells with large numbers of mitochondria still function if irradiation compromises a substantial proportion (196). Mitochondrial DNA appears more susceptible to damage by IR and chemically-induced oxidative stress (182). This DNA codes the subunits of the electron transport chain (ETC) (197-200) which is an important site of superoxide production (201). Multiple experimental studies have shown that IR directly impacts ETC complexes and ATP synthesis, disrupting oxidative phosphorylation (182).

Leach et al showed that when osteosarcoma cells lacking mitochondrial DNA were irradiated there was no increase in secondary redox disruption, supporting a central role for mitochondria (202). Leach et al also found the calcium binding protein calbindin limited redox changes, suggesting that calcium played a role in the secondary response (202). Signalling between mitochondria and the nucleus may also be affected by IR; elevation in a marker of nuclear DNA damage was shown five minutes after nuclear targeting with microscopic irradiation, compared to three hours after cytoplasmic irradiation (202). Furthermore, the bystander effect was not observed when cells deficient in mitochondrial DNA were used suggesting mitochondrial function was an essential element of intercellular signalling. Richardson and Harper found that uncoupling the ETC lowered oxidant production and decreased radiosensitivity especially for hypoxic tumours (203). They demonstrated that damage to oxygenated tissue is related to mitochondrial oxygen consumption and the production of oxidants, and argued the primary radiation targets in oxygenated tissues are mitochondria that in turn target nuclear DNA.

Cellular antioxidant systems are responsible for maintaining redox homeostasis and protect against the effects of oxidative stress, including DNA damage (204). As such, overexpression of these endogenous antioxidants can protect cells from radiation-induced injury. This effect was most significant for MnSOD, slight for glutathione peroxidase, while copper-zinc superoxide dismutase (Cu,Zn-SOD) appeared to make no difference (205-207). The fact that MnSOD resides in mitochondria while Cu,Zn-SOD is in the cytosol is consistent with mitochondrial damage playing a key role in radiation-induced injury.

The role of dietary antioxidants in cancer therapy remains unclear (208). In contrast to cellular enzymes, it is difficult for small molecule oxidant scavengers to reach sufficient concentrations at intracellular sites to have significant impact. Antioxidant supplementation

has been reported to reduce side effects from chemotherapy (209), but this could potentially result in decreased treatment efficacy by reducing that oxidative damage that triggers cancer cell death. For example, the DNA-damaging ability of phenolic phytochemicals was shown to be inhibited by ascorbate and N-acetylcysteine in colon cancer cells (210) suggesting that antioxidants can modulate the response to DNA-damaging agents. There is currently no defined role for antioxidant supplementation in colorectal cancer patients.

Differences in radiosensitivity have been found in cells of variable peroxiredoxin expression, and a protective effect against radiation has been found with increased Prx1, Prx2 and Prx4 expression (211). Peroxiredoxins were proposed as a novel target for radiotherapy by Zhang et al (211), on the basis of expression induction by IR in a wide range of cell lines, including human HT29 colon cancer cells, as well as well as tissue including colorectal and non-colorectal tumours (212-217), and an association between expression status and radiosensitivity of tumour cells including decreased radiosensitivity after knockdown of peroxiredoxins (217, 218).

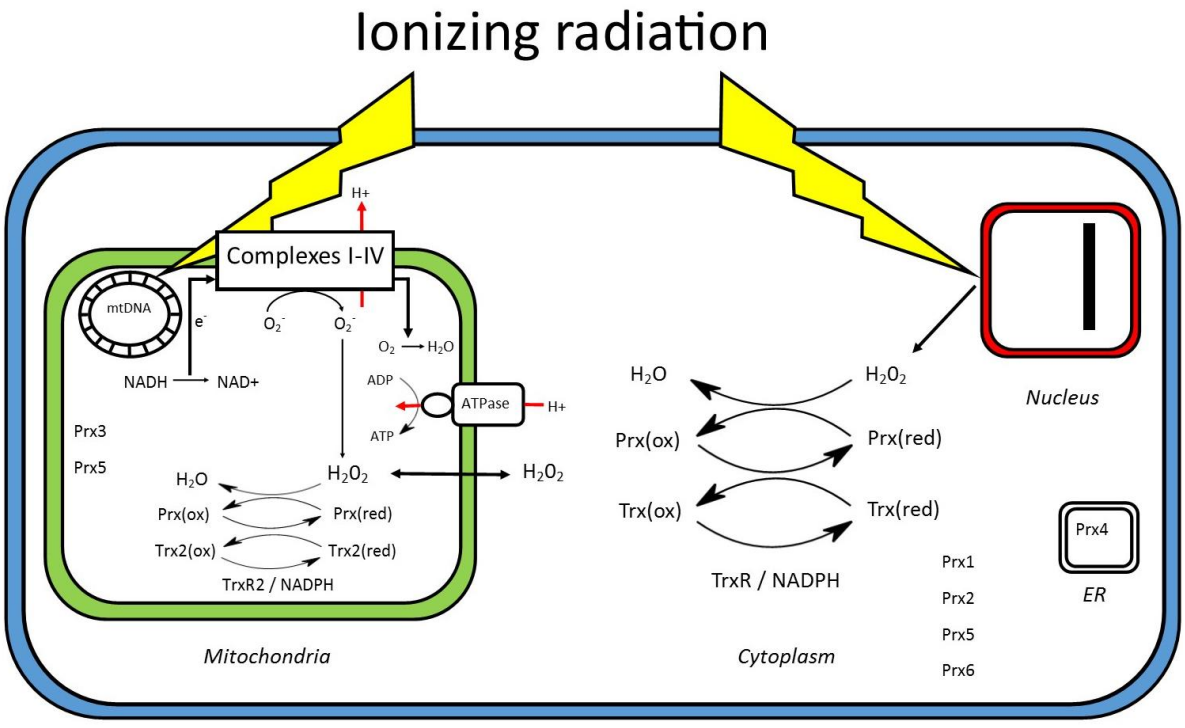

Figure 3.1. Overview of disrupted redox homeostasis and peroxiredoxin activity following exposure of cells to ionising radiation.

3.4 Peroxiredoxins and Colorectal Cancer

There have been several studies examining peroxiredoxin expression in CRC (Table 3.1); with expression of all six peroxiredoxins reported to be increased (219). We review these studies, with particular focus on radiosensitivity for each peroxiredoxin.

Table 3.1. Peroxiredoxin expression and associations with radiosensitivity and prognosis of colorectal cancer

Gene name	Expression in CRC	Radiosensitivity	Prognostic indicator	Predictor of pathological tumour response	Ref
PRDX1	↑	↑ expression → ↓ radiosensitivity	Yes	↑ expression → ↓ response	(220)
PRDX2	↑	↓ expression → ↑ radiosensitivity	Yes	-	(221-226)
PRDX3	↑	-	-	-	(227)
PRDX4	↑ / ↓	-	Yes	Yes	(219, 228, 229)
PRDX5	↑	-	-	-	(230)
PRDX6	↑	-	-	-	(219)

Legend: ↑ = increased, ↓ = decreased, → = association between expression and radiosensitivity or tumour response.

3.4.1 Peroxiredoxin 1

Prx1 expression is increased in CRC and has been suggested as a prognostic and predictive biomarker for rectal cancer on the basis of both in vitro and in vivo studies conducted by Chen et al (220). Prx1 expression as evaluated by immunohistochemistry (IHC) was significantly associated with a poor pathological response rate for 120 human subjects with rectal cancer treated with radiotherapy, with a response rate of 43.6% when there was negative staining and 20% when there was positive staining; this effect was accentuated when p53 staining was negative. Prx1 suppression by a Prx1 silencing vector increased radiosensitivity of HT-29 and HCT-116 colon cancer cell lines and inhibited tumour growth in a mouse model. Prx1 expression was also a significant predictor of disease free survival (DFS) in the group of patients who were p53 negative. The author's conclusion that Prx1 expression was associated with both poorer response to treatment and poorer prognosis appears justified but little work has been done to further investigate this.

3.4.2 Peroxiredoxin 2

Peng et al demonstrated that both Prx2 mRNA and protein content was higher in CRC cell lines than normal colonic epithelial cells, and Prx2 expression was significantly upregulated in human CRC tissue compared with adjacent non-cancerous tissue (221). They also assessed clinicopathological correlation and identified an association between increased Prx2 expression and poor histological differentiation, advanced local invasion, lymph node metastases and advanced tumour node metastasis stage, as well as shorter DFS, suggesting it may have a useful role as a prognostic marker for CRC (221). In support of Prx2 expression as a stimulator of cancer progression, when a tumour knockdown of Prx2 was performed in a mouse model it was found to inhibit CRC cell growth, and when Prx2 silencing was performed in both a polyposis mouse model and human CRC cell lines, mouse polyposis was decreased by a reduction in beta-catenin as an end-point, and beta-catenin levels are reduced in the cells in which Prx2 is silenced (224). This suggests a mechanism of action involving the canonical Wnt signalling pathway. Although this is one of many molecular pathways in CRC development, the canonical Wnt pathway is disrupted in familial adenomatous polyposis due to germline mutations of the adenomatous polyposis coli gene, and commonly disrupted in sporadic CRC development. This raises the exciting possibility of therapeutic agents to limit polyposis progression in patients with familial adenomatous polyposis, as well as to modify risk of sporadic CRC. Lu et al also found that Prx2 was upregulated in CRC, and contributed to CRC cell survival by protecting cells from oxidative stress (222).

In contrast to the above findings, an earlier study by Ji et al (225) examined mRNA and protein expression of Prx2 in CRC tissue of 137 patients, and found lower Prx2 expression was associated with poor differentiation, advanced cancer stage and poorer survival. They also looked for a correlation between serum Prx2 and OS or DFS and found none (225). There is no clear explanation of the difference between this study and that of Peng et al (221).

Silencing of Prx2 expression has been shown to sensitize colon cancer cell lines to 5-fluoruracil by facilitating cell death and apoptosis (226), and also to sensitize colon cancer cells to IR (217). Despite promising work with cell lines and xenograft models, there has been no investigation of Prx2 as a biomarker for pathological response to radiotherapy for rectal cancer in human subjects in vivo.

3.4.3 Peroxiredoxin 3

Prx3 is the only mammalian peroxiredoxin that is present exclusively in mitochondria (231). Song et al collected tumour tissue from eight patients with colon cancer and investigated Prx3 expression using immunofluorescent and quantitative techniques (227). They found increased expression in colon cancer stem cells compared with normal colorectal tissue stem cells, and that cell death was not increased with escalating 5-fluoruracil dosing in colon cancer stem cells, showing some resistance to chemotherapeutic action. This suggests a cell survival advantage associated with Prx3 expression. The effect of Prx3 expression on radiosensitivity in CRC has not been examined, despite the importance of mitochondria in the response to IR.

3.4.4 Peroxiredoxin 4

The endoplasmic reticulum protein Prx4 has also been linked to CRC; Prx4 expression was higher in CRC tissue than normal colorectal tissue assessed with IHC and qPCR techniques, and increased Prx4 expression also correlated with negative clinical factors including depth of invasion and stage (228). In contrast, a small study that looked at peroxiredoxins in eight patients with CRC found Prx4 trending towards a lower positivity rate in CRC tumour tissue than normal controls and had no association with clinical stage or lymph node metastases (219). Prx4 expression by western blotting was slightly higher in normal control tissue than CRC tissue. Both these studies included small numbers of samples and statistical significance for positivity was not achieved in the second study, so this inconsistency may be a reflection of inadequate sample size.

An exploratory study investigating novel markers predicting pathological response to chemoradiotherapy for rectal cancer using a 2D-DIGE (difference gel electrophoresis) quantitative proteomic approach in 35 patients with rectal cancer found higher Prx4 expression in pre-treatment tumour samples in poor responders to chemoradiotherapy, suggesting a potential role as a predictive biomarker of response to chemoradiotherapy for rectal cancer (229). There were no differences seen in any of the other peroxiredoxins in this study.

3.4.5 Peroxiredoxin 5

There is limited literature available on the role of Prx5 in CRC, but a recent study demonstrated increased expression of Prx5 in colon cancer cell lines is associated with cell proliferation, migration and invasion, while decreased expression had the inverse effect (230). This study also found enhanced tumour growth with increased expression of Prx5 in a xenograft mouse model. There are no reports relating to Prx5 in human CRC.

3.4.6 Peroxiredoxin 6

Wu et al demonstrated Prx6 expression positivity in 56% of CRC tissue vs 12.5% of normal control tissue, and significantly higher expression in CRC tissue on western blotting, but no association with clinical stage or lymph node metastases (219). There are no other reports linking Prx6 to CRC development, progression or treatment response.

3.5 Peroxiredoxins as Prognostic and Predictive Biomarkers

Tumour biology is often the most important determinant of patient outcome, and tumour features can be useful for predicting the natural history of CRC. There is significant evidence to support an association between increased Prx2 expression and poor prognostic factors such as more advanced tumour stage and decreased survival. Prx1 has also been associated with clinical outcomes. Peroxiredoxins may be one marker of the underlying tumour biology as redox homeostasis itself is critical to cell survival.

The studies described above suggest that peroxiredoxin expression level may have a role in predicting radiosensitivity and/or chemosensitivity for CRC. The majority of this evidence is based on work with cell lines or animal models, with limited evidence of in vivo response to treatment in human subjects. Only Prx1 and Prx4 have been linked to radiotherapy response for rectal cancer in vivo, and each by a single study. Prx2 has not yet been linked to response to radiotherapy for rectal cancer but an apparent role in the development, progression and in vitro response to chemo/radiotherapy of CRC makes it a good candidate for further investigation.

Peroxiredoxins are sensitive markers of cellular redox homeostasis. Typical 2-Cys peroxiredoxins can be present in oxidized homodimers (~40kD) or reduced monomers (~20kD), with the oxidized form accumulating in cells due to either increased rates of

hydroperoxide generation or limitations in the rate of reduction of the oxidized forms (193). A simple method of measuring the oxidized and reduced forms of peroxiredoxins exists by western blotting of samples in which proteins have been separated by non-reducing polyacrylamide gel electrophoresis, and the relative ratio of oxidized and reduced peroxiredoxin calculated. This methodology has been shown to be valuable in measuring oxidative stress in erythrocytes and cardiac tissue (193) and in cultured cells treated with cytotoxic agents such as auranofin and phenethyl isothiocyanate (192, 232). In the studies to date investigating peroxiredoxins in CRC, total peroxiredoxin expression has been assessed, but not the redox status. Indeed, no comprehensive analysis of peroxiredoxin redox status in tumour material has been reported.

3.6 Peroxiredoxins as Therapeutic Targets

Work on expression silencing in laboratory models suggests peroxiredoxin inhibition as a possible therapeutic strategy. Various peroxiredoxin inhibitors have been described (233-235), including inhibitors of mitochondrial Prx3 (236), but there have been no studies specifically addressing this in CRC. Inhibition of peroxiredoxins in CRC could result in either increased oxidative stress during IR, and may even have direct anti-tumour activity. Another potential therapeutic target is the thioredoxin system, which is important in maintaining peroxiredoxins in their reduced form. Thioredoxin reductase inhibitors such as auranofin have been shown to result in cell death due to mitochondrial dysfunction and hydrogen peroxide accumulation in the context of neurological disorders (237) and have been proposed as anti-cancer agents based on upregulation in advanced malignancy and impairment of tumour growth in human tumour xenografts in mouse models (238). A thioredoxin-1 inhibitor has been shown to inhibit growth and progression of CRC cell lines (239), and while there has been no examination of radiosensitivity this would be worthy of investigation.

3.7 Summary and Discussion

Predicting radiation sensitivity of rectal cancer carries enormous clinical significance, particularly in the setting of an evolving organ preservation approach to management. Tools to assist in management decisions regarding organ preservation strategies would be of

important clinical value. Mitochondrial function including redox homeostasis is integral in the cellular response to IR, and peroxiredoxins are important players in these systems.

There is evidence of increased expression of all six peroxiredoxins in CRC, albeit with some inconsistencies among reported associations for Prx2 and Prx4. These inconsistencies indicate a need for further research. Prx1, Prx2 and Prx4 appear the most promising as prognostic indicators and/or predictive biomarkers of response to radiotherapy for CRC based on the available evidence. Prx3, Prx5 and Prx6 have limited data to support a role as markers, but what is available does suggest increased expression in CRC and the role of these enzymes in CRC is in need of further investigation. Prx3 is of particular interest as the only peroxiredoxin present exclusively in mitochondria, given the central role of mitochondria in the response to IR.

The potential to increase cancer cell death and the chance of pathological complete response from radiotherapy for rectal cancer is real. Neoadjuvant radiotherapy is commonly given with fluoropyrimidine-based therapy as a radiosensitiser. It is possible modulation of the peroxiredoxin/thioredoxin system could improve response to chemoradiotherapy for rectal cancer by acting on both radiotherapeutic and chemotherapeutic pathways.

It is difficult to compare the radiotherapy response for colon and rectal tumours due to the different uses of radiotherapy in the two sites, and because the clinical behaviour of colon and rectal cancer differs significantly despite histological similarity. The clinical utility of radiotherapy is much greater for rectal cancer than colon cancer, therefore further investigation of the radiotherapy response associated with peroxiredoxin expression and redox status would be best studied in human subjects with rectal cancer if the potential for clinical translation is to be maximized.

4 Markers of Oxidative Stress as Novel Predictors of the Response to Radiotherapy for Rectal Cancer

4.1 Introduction

Extensive research has been performed attempting to identify predictive biomarkers of the response to radiotherapy for rectal cancer (Chapter 1). Common approaches have included genetic sequencing and immunohistochemistry to investigate the presence or absence of a specific genetic sequence or quantification of a specific protein. Despite the central role of oxidative stress in the cytotoxicity of ionising radiation, there has been little research investigating the redox status of tumours as a predictive biomarker. Complex dynamic systems can be difficult to investigate due to a large number of variables and incomplete understanding of the system of interest, particularly redox homeostasis. In this chapter three markers of oxidative stress were chosen for assessment: peroxiredoxin 2 and peroxiredoxin 3 oxidation, and protein carbonyls. None of these have been previously investigated as predictors of response to radiotherapy for rectal cancer.

The background and rationale for investigation of peroxiredoxins as predictors of radiation response for rectal cancer is outlined at length in Chapter 3. In summary, oxidative stress is a major mode of tumour cell death in radiotherapy. Peroxiredoxins are a family of thiol-dependent antioxidants and include six different types in human cells, in which they are abundant. Peroxiredoxins reduce reactive oxygen species (ROS) with a particular effectiveness for hydrogen peroxide, and are therefore central in maintaining redox homeostasis in cells. The catalytic activity of peroxiredoxins is dependent on an active cysteine residue that is oxidised to a sulfenic acid (240). Peroxiredoxin oxidation is a reversible process and oxidised peroxiredoxins are reduced by thioredoxin or glutaredoxin meaning the enzymes are recyclable. Oxidised and reduced forms of peroxiredoxins have been used as markers of oxidative stress in biological systems as accumulation of the oxidised form occurs with sustained oxidative stress (193). Oxidation of peroxiredoxins occurs rapidly when exposed to the atmosphere, therefore peroxiredoxins need to be trapped in their redox state prior to cell lysis by use of an alkylating agent such as N-ethylmaleimide (NEM). The ratio of oxidised dimers (~42kD) to reduced monomers (~21kD) can be seen on western blotting of non-reducing gels (Figure 4.1).

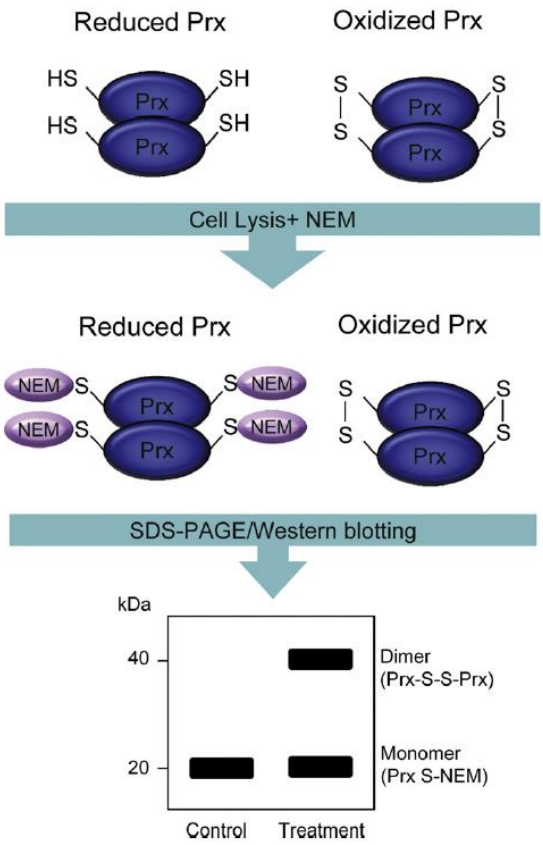

Figure 4.1. Action of N-ethylmaleimide in trapping the oxidation state of typical 2-Cys peroxiredoxins and visualisation on western blotting (reproduced from reference (193))

Peroxiredoxin 2 (Prx2) and peroxiredoxin 3 (Prx3) are present in different cellular compartments (cytosol and mitochondria respectively) and methods of western blotting for cell lysates have been shown to be successful previously as indicators of increased oxidative stress in cell culture (241) and isolated rat hearts (242). Although peroxiredoxin 1 has been suggested as a possible candidate for further research in previous studies examining radiosensitivity of CRC (220), it is from the same cellular compartment as peroxiredoxin 2. Because western blotting is very antibody dependent, peroxiredoxin 2 was chosen due to proven past performance in the laboratory and lack of evidence to suggest there should be a major difference between peroxiredoxin 1 and peroxiredoxin 2. No work has been published using human tissue. Mitochondria are important sources and targets of oxidative stress, as discussed in Chapter 3. Prx3 is a mitochondrial enzyme, and its activity may be increased by either specific upregulation or due to an increase in numbers of mitochondria. The number of mitochondria present in a cell is dynamic, with production (mitogenesis) and destruction (mitophagy) varying according to biological need.

Protein carbonyls are biomarkers of global protein oxidation that are generated by several different mechanisms during oxidative stress, including direct oxidation of amino acids, cleavage of the protein backbone and lipid peroxidation (243). A protein carbonyl is formed when an aldehyde or ketone group is added to a side chain, most commonly on arginine, proline, threonine and lysine residues, forming a chemically stable compound (243). By introducing a detectable functional group into an oxidised protein, for example by derivatisation with dinitrophenyl hydrazine (DNP), protein carbonyls can be measured. Carbonyls have been demonstrated to be markers of oxidative stress (244) and have been investigated in numerous translational studies, including inflammatory conditions such as acute pancreatitis (245) and ulcerative colitis (246), infectious diseases such as leptospirosis (247) and colorectal cancer (246). They have not been investigated as a predictor of response to radiotherapy for rectal cancer.

4.2 Hypothesis

Baseline oxidative stress of tumour cells predicts sensitivity to radiotherapy for rectal cancer.

4.3 Objectives

The primary objective was to determine the relationship between markers of oxidative stress and the response to chemoradiotherapy for rectal cancer.

The secondary objectives were to assess differences in markers of oxidative stress between normal rectal mucosa and rectal tumour tissue, and to assess changes in markers of oxidative stress in rectal tumour and normal rectal epithelium after radiotherapy.

4.4 Methods

This prospective observational pilot study was conducted from February 1 2018-January 30 2019. Patients treated for rectal cancer at Christchurch Hospital were recruited by screening colonoscopy lists and the colorectal multi-disciplinary meeting (MDM), and liaison with clinicians.

Inclusion required a confirmed rectal tumour in adult patients (>18 years of age). Patients were excluded if repeat sigmoidoscopy was required and posed more than minimal clinical risk e.g. frail patient, anticoagulants or other medications requiring cessation.

4.4.1 Clinical Protocol

4.4.1.1 Staging

Routine staging investigations were performed with magnetic resonance imaging (MRI) of the pelvis for loco-regional assessment and computed tomography (CT) of the chest, abdomen and pelvis to assess for distant metastases. Additional imaging such as MRI liver or PET/CT scan was performed when clinically indicated. Imaging interpretation was reported by a consultant radiologist in a synoptic manner based on the American Joint Committee on Cancer (AJCC) TNM staging system. Serum carcinoembryonic antigen level (CEA) was obtained at diagnosis.

4.4.1.2 Treatment

The management of each participant was determined by the clinical team with input from the MDM and without consideration to the study. This included recommendations regarding radiotherapy, chemotherapy and/or surgery.

Radiotherapy for rectal cancer is relatively standardised in Christchurch Hospital. Short-course radiotherapy includes 25 Gray in five fractions with surgery usually performed within 7-10 days. Long-course radiotherapy includes 45 Gray in 25 fractions of whole pelvis radiotherapy with a 5.40 Gray boost to the tumour in a total of 28 fractions, and surgery is performed 8-12 weeks later. Concurrent chemotherapy is given with long-course radiotherapy (nCRT) if the patient does not have precluding co-morbidity, most commonly in the form of oral capecitabine. Organ preservation using a watch-and-wait strategy (i.e. deferred surgery with surveillance of a complete clinical response) is not routine, but is occasionally considered based on individual patient risk profile.

Rectal resection was carried out by a qualified colorectal surgeon at Christchurch Hospital (Canterbury District Health Board). Total mesorectal excision (TME) is employed routinely for LARC and may be open, laparoscopic or a trans-anal/laparoscopic combined technique. Surgical options include rectal resection with sphincter preservation with or without anastomosis (anterior resection), or en-bloc sphincter-excision by abdominoperineal resection. The timing of mesenteric vascular ligation was determined by the operating surgeon but is commonly performed early in the operation.

4.4.2 Recruitment

All patients gave written informed consent. Patients were recruited by one of two possible pathways. Figure 4.2 demonstrates the timeline of each pathway in relation to the common clinical pathway. The pre-diagnostic pathway required a two-stage consent process. Patients with high suspicion of rectal cancer (determined by JF) were approached prior to diagnostic colonoscopy. General consent was obtained for additional colorectal biopsies for research purposes. If rectal cancer was confirmed, the patient was approached with further information and an additional consent form completed for full enrolment in the study. The post-diagnostic pathway was used for patients with a confirmed diagnosis of rectal cancer. If flexible endoscopy was planned for clinical reasons (e.g. to confirm histological diagnosis or for operative planning prior to neoadjuvant radiotherapy) biopsies were taken at this time. If additional flexible endoscopy was not planned rigid sigmoidoscopy and biopsy was performed.

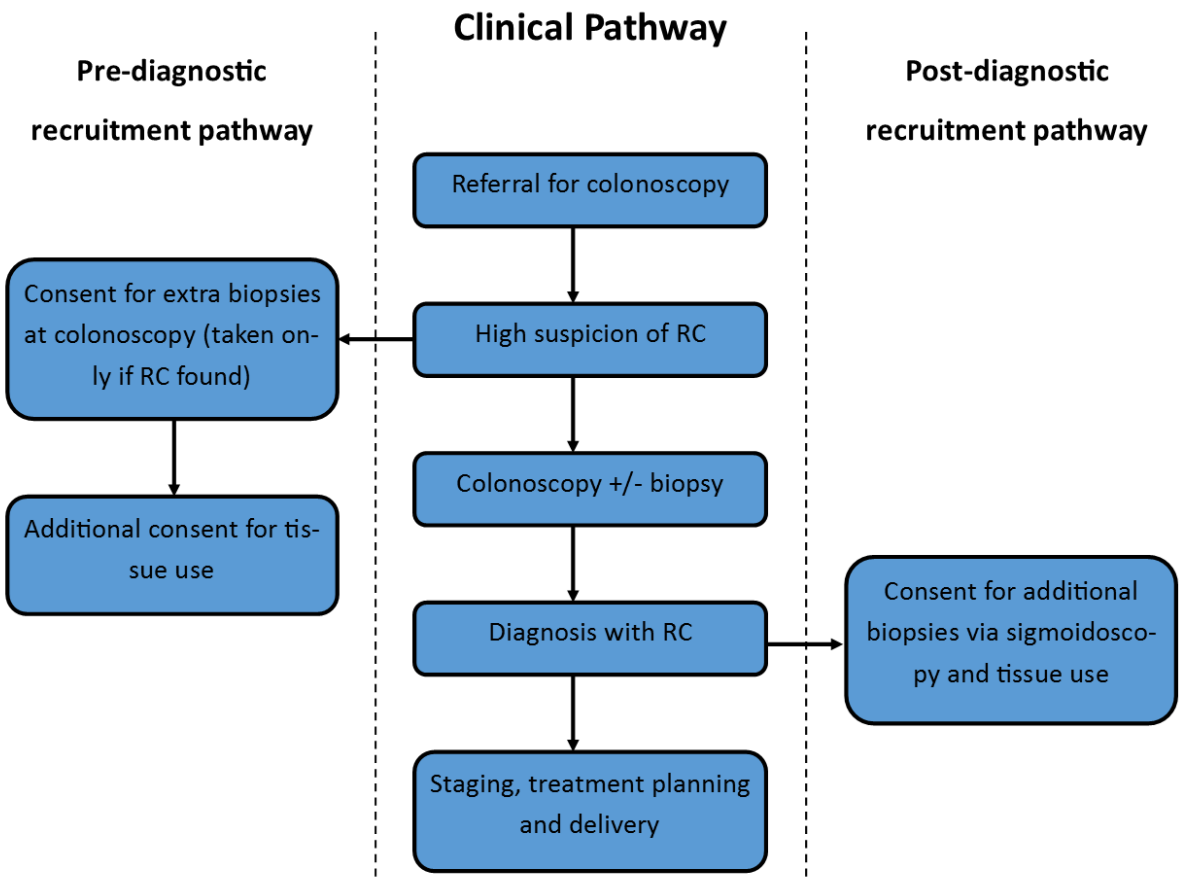

Figure 4.2. Timeline of recruitment pathways

4.4.2.1 Clinicopathological Data Collection and Follow-up

Clinical, pathological and radiological data was recorded for each participant. The AJCC TRG was recorded for each patient treated with radiotherapy who underwent surgical resection. Patients were followed until the day of surgery, or until the first repeat sigmoidoscopy and biopsy in the event of a complete clinical response (cCR) for which resection was not performed.

4.4.3 Biopsy and Tissue Processing Protocol

4.4.3.1 Endoscopic Biopsies

Biopsies were taken from a peripheral location within the tumour, avoiding ulcerated and necrotic areas. Biopsies of normal rectal mucosa were taken at least 5 cm from the tumour. Normal mucosa was biopsied first to prevent theoretical chance of seeding cancer cells to normal tissue, or from an anatomical location expected to be removed at surgery. JF directed the endoscopist to ensure optimum tissue sampling, with the exception of two cases when the task was delegated to a suitably-qualified individual. Up to ten biopsies were taken from each participant using flexible endoscopic biopsy forceps. Due to the larger size of biopsies taken with rigid sigmoidoscopy, approximately six biopsies were necessary to obtain a similar amount of tissue as obtained from 10 flexible endoscopic biopsies.

4.4.3.2 Tissue Collection Protocol

Both tumour and normal tissue biopsy specimens were placed in 1.6 mL Eppendorf tubes with either 1 mL phosphate buffered saline (PBS) or 1 mL PBS with 100 mM NEM immediately after the biopsy was taken. The time from biopsy to immersion in buffer was less than 30 seconds for pre-treatment biopsies; for resection specimen's tumour and normal rectal mucosa tissue was processed within 20 minutes of extraction of the surgical specimen using procedures established for the Cancer Society Tissue Bank (CSTB). JF attended the operating theatre to expedite transfer of the surgical specimen to Anatomical Pathology when possible. Surgical specimens were examined by the consultant pathologist or registrar on duty, and both tumour and normal tissue surplus to diagnostic requirements was obtained for the study. The PBS + NEM buffer was warmed to room temperature prior to biopsy collection, and maintained at room temperature while the PBS samples were maintained below 4^{o}C on wet ice. Additional samples of normal and tumour tissue from pre-

treatment biopsy were snap frozen in the endoscopy suite using dry ice and stored in the CSTB.

4.4.3.3 Tissue Lysate Preparation – Method A

1. On arrival at the laboratory the tissue was transferred to new Eppendorf tubes containing approximately 250 µL of the same buffer (PBS or PBS + 100 mM NEM) per endoscopic biopsy.
2. Biopsy material was homogenised with a glass hand homogeniser in an Eppendorf tube as soon as possible, rinsing the homogeniser with water then PBS between samples to prevent cross-contamination.
3. Samples were incubated at room temperature for 15 minutes after homogenisation then lysis buffer added and placed on wet ice. Lysis buffer contained 40µL 20% sodium dodecyl sulfate (SDS), 100 µL 10% NP-40 detergent, 100 µL 10% sodium deoxycholate and 25 µL 50x complete protease inhibitor for each 1000 µL of buffer.
4. Samples were sonicated on ice using three five second bursts with 30 second pauses between to prevent samples from overheating.
5. Centrifuged at 12,000 g for 20 minutes at 4 ^{o}C.
6. Supernatant was collected into a new 1.6 mL Eppendorf tube and frozen, pellet discarded.
7. Samples aliquoted on first thawing after measuring protein content and subsequently stored in -80 ^{o}C freezer.

4.4.3.4 Tissue Lysate Preparation – Method B

Later samples were homogenised using the Precellys Evolution Cryolys bead homogeniser (Bertin Instruments) after determining equivalent results with both methods. Steps 1-3 were identical to Method A and subsequent steps are below.

1. Placed in 2 mL reinforced tube with fresh buffer. Ceramic 2.8 mm beads (CK28R) used on advice from a Bertin Instruments scientific representative.
2. Spun for three 15 s cycles with 30 second gap between each, maintained at 4 ^{o}C.
3. Lysis buffer added.

4. Centrifuged for five minutes at 4 ^{o}C to remove bubbles then transferred to Eppendorf and steps 5-7 in Method A followed.

To compare the two methods of tissue homogenisation, the same biopsy tissue was processed by the two methods (Method A and Method B) in parallel. Comparison of '9R' resection specimen processed by both methods was found to have similar total protein concentration and a western blot demonstrated similar %Prx2ox (not shown).

4.4.3.5 Protein Quantification

Protein concentration was measured using the DC™ Protein Assay (Bio-Rad). The DC™ Protein Assay is a detergent compatible colorimetric assay for protein concentration modified from the Lowry assay. Absorbance was measured in a microplate reader and related to a standard curve constructed on each occasion using bovine serum albumin (BSA). The microassay method was used in this study due to limited sample volumes available. Protein quantification was performed in duplicate or triplicate.

4.4.3.6 Gel Protein Electrophoresis and Western Blotting

Tissue lysate was used to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a non-reducing gel. Non-reducing gels were used because the ratio of oxidised to reduced forms of peroxiredoxins was the main outcome of interest, and a reducing gel would prevent assessment of this. Mini-PROTEAN® TGX™ precast gels (Bio-Rad Laboratories, Inc) were used. Protein was transferred from the gel to a polyvinylidene difluoride (PVDF) membrane and then immunostaining performed using the western blot technique. Membranes were blocked for one hour (using 5% milk or 5% BSA) then the primary antibody was applied and incubated overnight at 4 ^{o}C (Table 4.1). The following day the membranes were washed with at least three washes of at least five minutes each with Tris-buffered saline and Tween 20 (TBST) then incubated in the secondary antibody for one hour at room temperature. Blots were washed again then developed using the Alliance Q9 Advanced Chemiluminescense imaging platform (UVItec Limited). Images were saved in TIF format.

Table 4.1. Details of western blotting methods including antibodies used.

	Protein loaded (µg)	**Primary antibody (concentration)**	**Secondary antibody (concentration)**	**Blocking agent**	**Incubating agent**
Prx2	5µg	Anti-peroxiredoxin 2 (C-terminal) rabbit polyclonal antibody, R8656, Sigma-Aldrich (1:20,000)	Goat anti-rabbit secondary antibody (Horseradish Peroxidase) (1:10,000)	5% w/v skim milk in TBST	5% w/v skim milk in TBST
Prx3	25µg	Anti-peroxiredoxin 3 rabbit polyclonal antibody, LF-PA0255, Abfrontier (1:7500)	Goat anti-rabbit (Horseradish Peroxidase) (1:10,000)	5% w/v BSA in TBST	5% w/v BSA in TBST

4.4.3.6.1 Quantification of Western Blots

Image J software (National Institute of Health and Laboratory for Optical and Computational Instrumentation, University of Wisconsin) was used to perform densitometry. Image J was used to open the TIF file and the bands of interest were isolated using the rectangle tool. For peroxiredoxins the same frame was used to measure oxidised and reduced bands for each sample. The band intensity peaks were plotted, the peaks were separated using the line tool, and the area under each peak measured.

For Prx2 and Prx3 the percentage of peroxiredoxin oxidation (%Prx_{ox}) was calculated using the equation:

$$\%Prxox = \frac{Density\ (oxidised\ peroxiredoxin)}{Density\ (oxidised\ peroxiredoxin) + Density\ (reduced\ peroxiredoxin)} \times 100$$

4.4.3.7 Protein Carbonyl Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

Protein carbonyls were measured using materials and methods available in a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioCell Protein Carbonyl Assay Kit). This ELISA involves derivatisation of protein samples with DNP which are then bound to the ELISA plate. The remaining process is outlined in Figure 4.3.

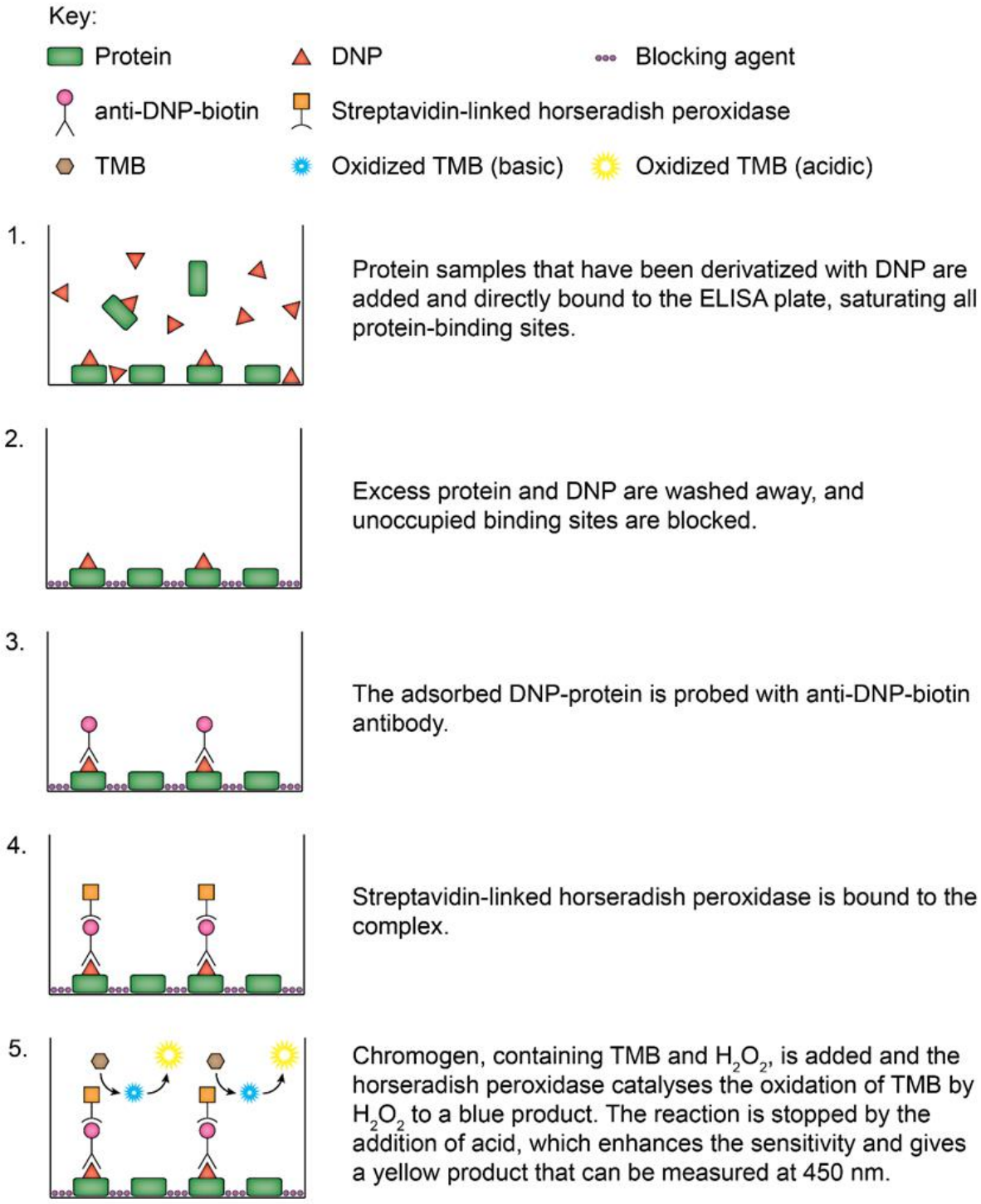

Figure 4.3. Outline of the protein carbonyl ELISA (reproduced from reference (248))

4.5 Results

23 patients were recruited during the study period. Diagnoses and treatment received are summarised in Table 4.2; three patients were found not to have rectal adenocarcinoma as initially suspected. One patient was excluded from analysis as they were diagnosed with locally advanced prostate carcinoma invading the lumen of the rectum. Two patients had advanced adenomas treated by local excision (ID 13 and ID 21); these patients were included

in comparison between tumour tissue and normal tissue but not in analysis related to treatment outcomes. For the 22 patients included, the median age was 67 (range 36-85) and 15 were male. All were of NZ European ethnicity except for one who was of Maori ethnicity. Pre-treatment clinicopathological data is reported in Table 4.2, with the sub-group used to assess predictors of response presented separately. Patients treated by nCRT and TME, or nCRT with a complete clinical response managed with a watch-and-wait strategy, were included in analysis to determine predictors of response to CRT (n=7).

Table 4.2. Treatment strategies employed for participants recruited

Diagnosis	Treatment	N (%)
Rectal adenocarcinoma	nCRT and TME performed	6
	nCRT with deferral of surgery due to complete clinical response	1
	Short-course radiotherapy and TME	3
	Straight to surgery, no neoadjuvant treatment	2
	Palliative treatment (no surgery)	2
	nCRT with TME planned	6
Rectal adenoma	Local excision	2
Prostate adenocarcinoma	Palliative	1

Table 4.3. Clinicopathological data for participants recruited

	N all patients (n=22)	**N (nCRT and TME) and cCR (n=7)**
Age (years) median (range)	67 (36-85)	64 (51-78)
Gender		
Male	15	4
Female	7	3
Ethnicity		
NZ European	21	7
Maori	1	0
BMI median (range)	28.2	29.6 (24.7-33.7)
Diabetes	2	1
Smoker (within 30 days)	3	0
Clinical T stage*		
T2	1	0
T3	13	5
T4	5	2
Clinical N stage*		
N0	9	1
N1	6	3
N2	5	3
Distance from anorectal margin on MRI (mm), median (range)	45 (0-150)	45 (0-130)
Differentiation*		
Well	0	0
Moderate	14	7
Poor	1	0
Mucinous histology	1	1
Position		
Anterior	4	1
Posterior	6	2
Lateral	2	0
Circumferential or near	8	3
Not circ, NOS		
Not available	1	0
	1	1
Tumour length on MRI (mm), median (range)	45 (26-75)	47 (32-66)
CEA, median (range)*	3.5 (0.7-26.7)	8.65 (1.3-26.7)

*rectal cancer only

Operative and pathological outcomes for patients who underwent TME after nCRT are presented in Table 4.4.

Table 4.4. Operative and pathological outcomes for patients who underwent TME following neoadjuvant chemoradiotherapy

	n=6 participants total
AJCC TRG	
0	2
1	2
2	2
3	0
R0	6
TME grade	
1	0
2	1
3	5
Differentiation	
Well	1
Moderate	3
Poor	0
Mucinous	0
LVI	0
PNI	0
(y)pN	
0	3
1	3
2	0
(y)pT	
0	2
1	0
2	2
3	2
4	0
Sphincter-preserving surgery	6

4.5.1 Assessment of Markers of Oxidative Stress

4.5.1.1 Peroxiredoxin 2 Oxidation

Western blotting for Prx2 yielded consistently good results with clear blots, minimal background and only 5 µg of total protein required (representative blot in Figure 4.4). Densitometry performed for duplicates of lysate samples and subsequent %$Prx2_{ox}$ calculated is shown in Figure 4.5 and Figure 4.6, demonstrating less than 10% variability for most samples.

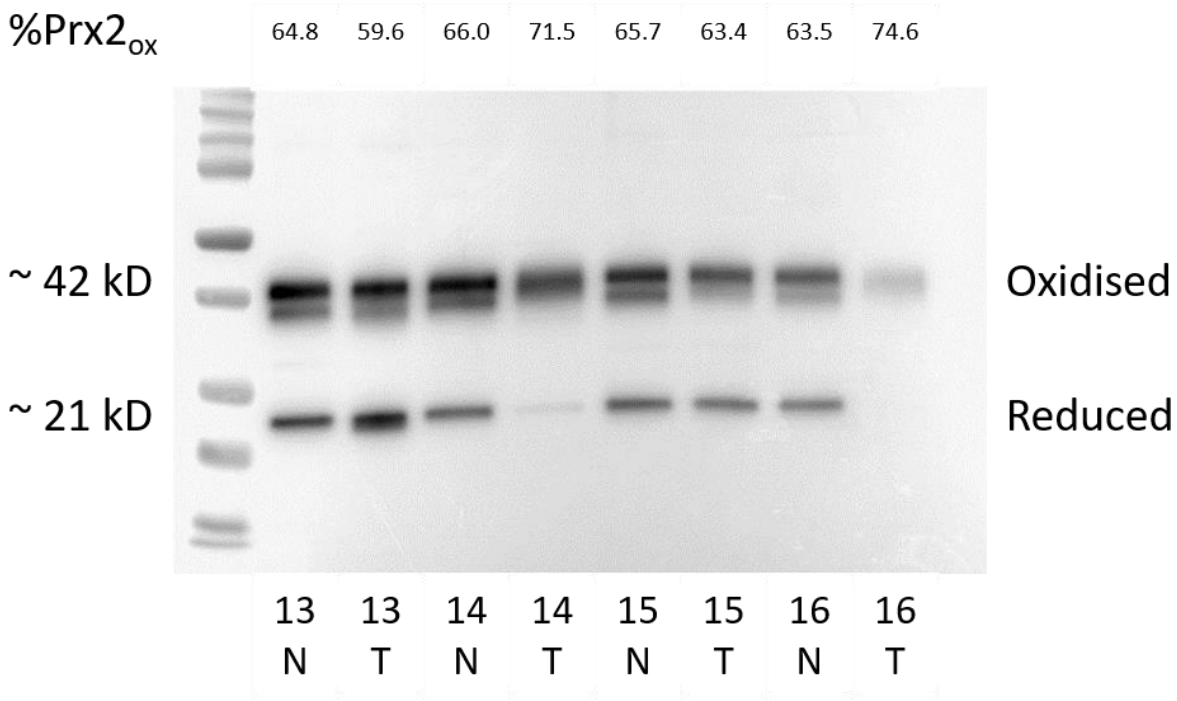

Figure 4.4. Representative western blot for peroxiredoxin 2 demonstrating oxidised and reduced forms

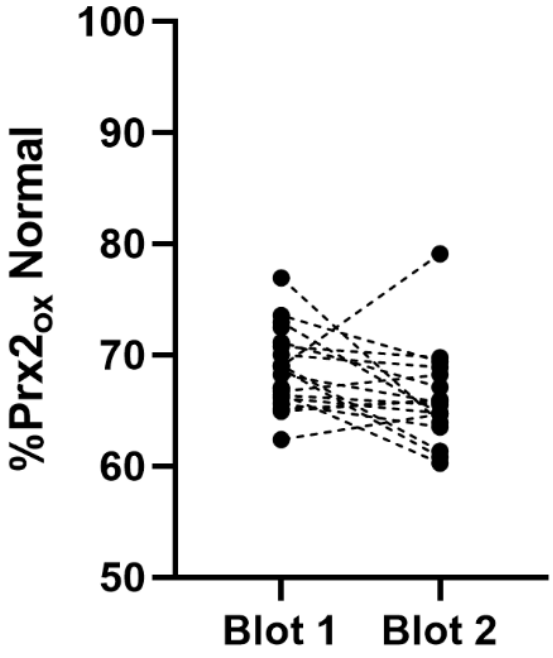

Figure 4.5. Variability in duplicate measurement of peroxiredoxin 2 percentage oxidation of normal samples

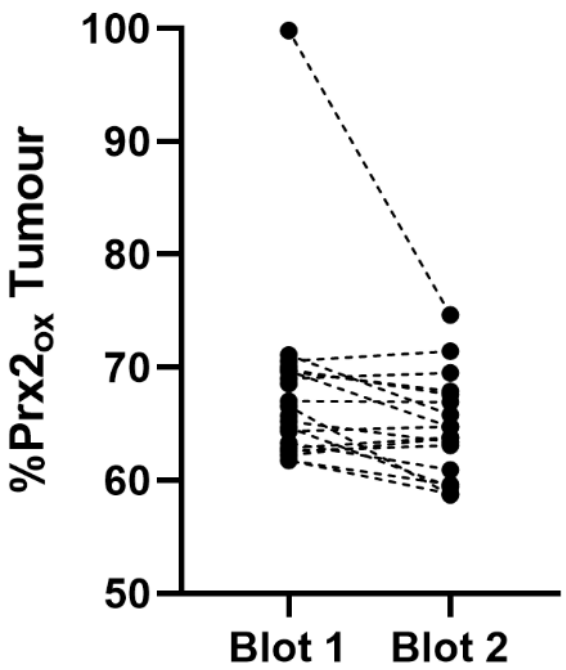

Figure 4.6. Variability in duplicate measurement of peroxiredoxin 2 percentage oxidation of tumour samples

4.5.1.2 Peroxiredoxin 3 Oxidation

In general Prx3 western blots were poorer quality with low signal, high background and the presence of a non-specific band at 15 kD despite best attempts at optimisation (representative blot in Figure 4.7, although non-specific band is not obvious in this figure). Methods of optimisation included increasing total protein to 25 µg, trying multiple different primary antibodies (Abcam Ab202120, ABfrontier AF17D2FF), increasing the concentration of the primary antibody, using a high-sensitivity chemiluminescent reagent and blocking and incubating in BSA instead of milk.

As shown in Figure 4.8 and Figure 4.9 the reproducibility was generally poorer, especially for tumour samples. $\%Prx3_{ox}$ was within a broader range (37.5% - 100%) than $\%Prx2_{ox}$.

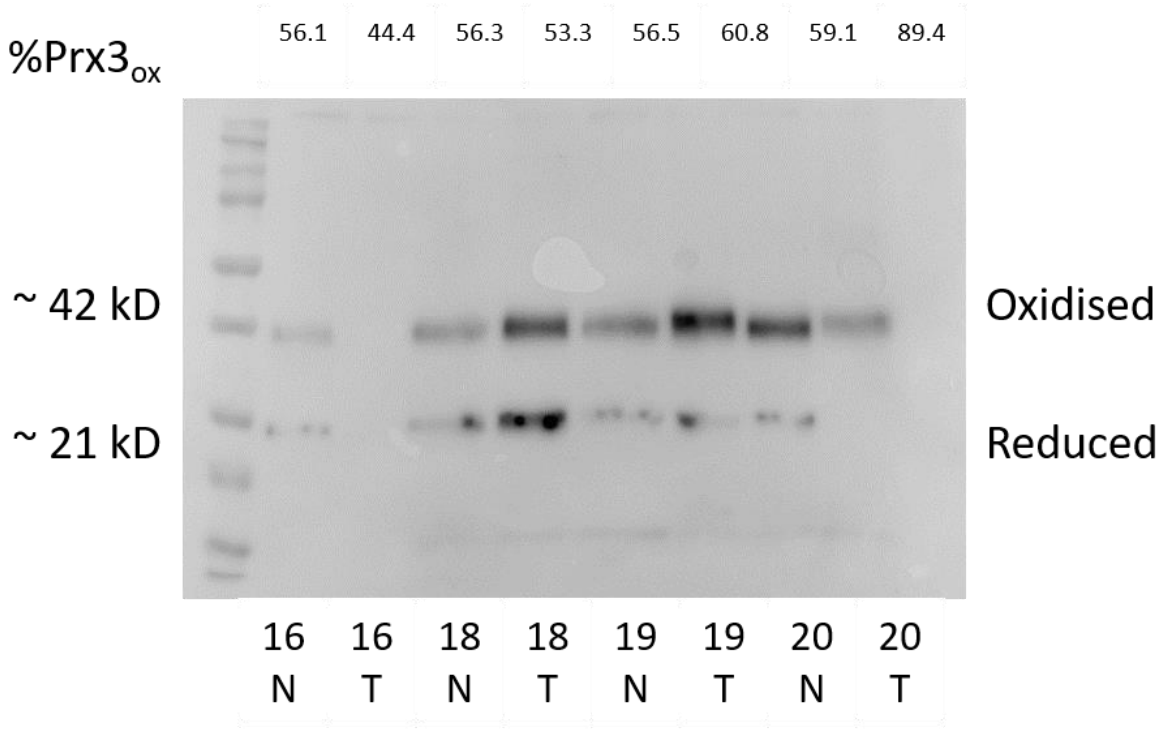

Figure 4.7. Representative western blot for peroxiredoxin 3 demonstrating oxidised and reduced forms

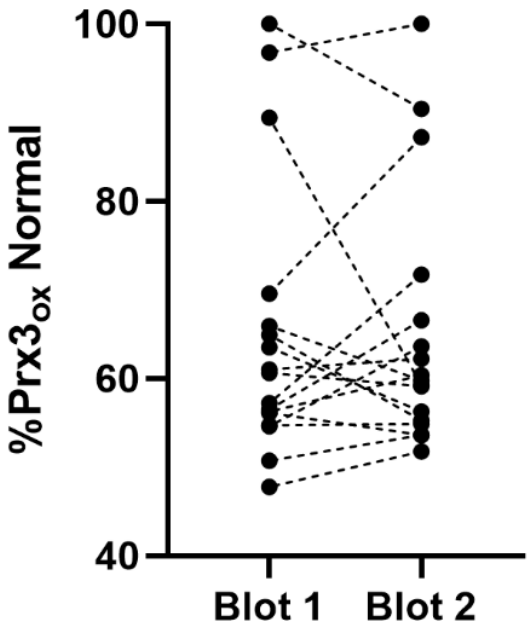

Figure 4.8. Variability in duplicate measurement of peroxiredoxin 3 percentage oxidation of normal samples

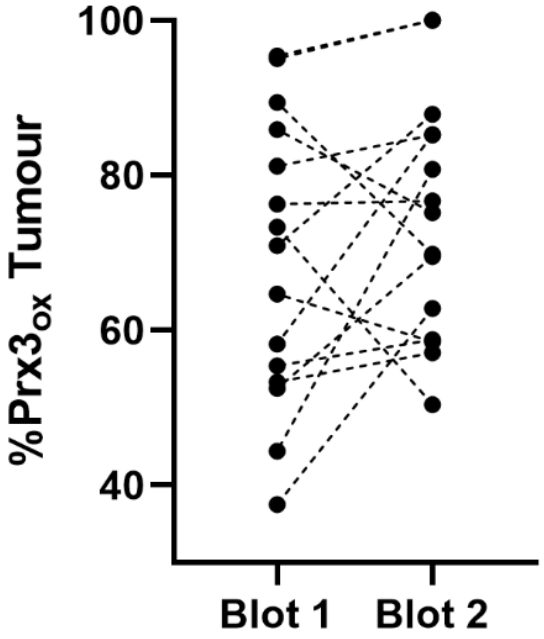

Figure 4.9. Variability in duplicate measurement of peroxiredoxin 3 percentage oxidation of tumour samples

4.5.1.3 Comparison of Normal Tissue and Tumour Samples

There was no significant difference overall between mean %$Prx2_{ox}$ in normal (mean 67.6, SD 2.96) and tumour samples (mean 66.6, SD 5.74) on a student's t test. There was a trend for tumour tissue oxidation to be less than normal tissue oxidation for most samples (Figure 4.10); 4/21 samples tumour was more oxidised compared with 17/21 normal tissue was more oxidised. Sample 16T is seen as an outlier with approximately 90% oxidation in Figure 4.10; this is likely a result of very low signal on western blotting (see Figure 4.4) therefore this sample was excluded from subsequent analysis.

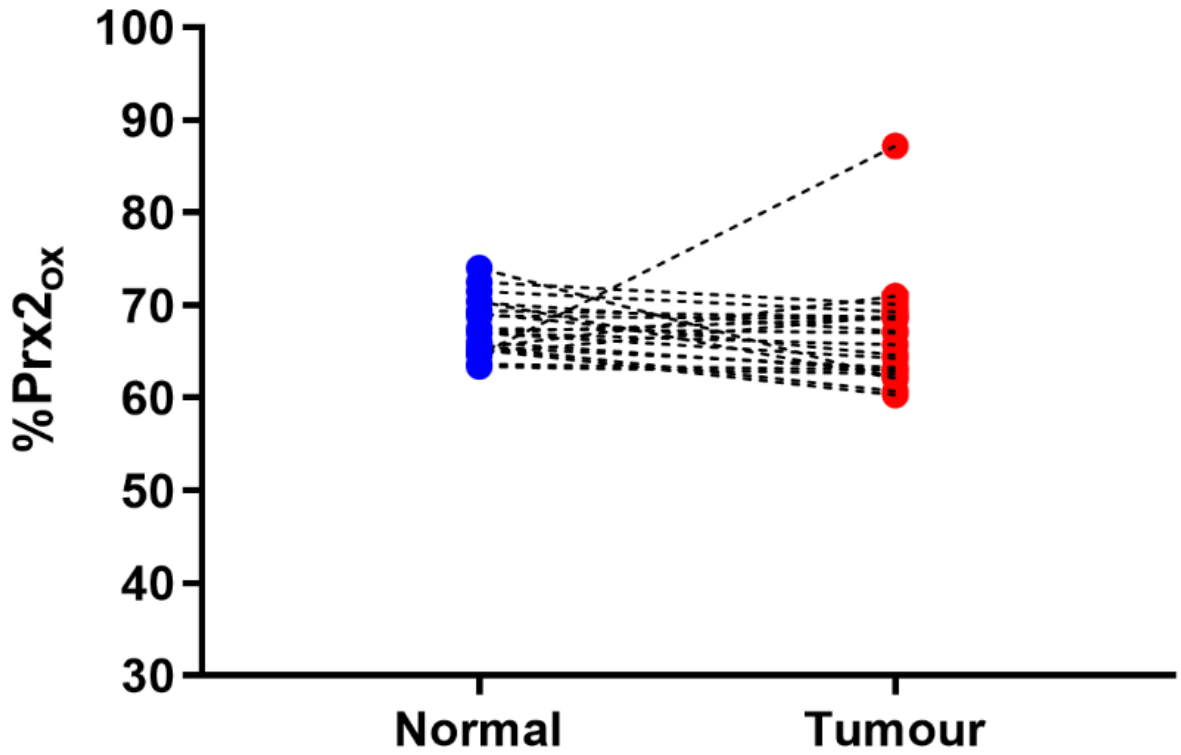

Figure 4.10. Relationship between peroxiredoxin 2 percentage oxidation for normal and tumour samples

It was possible to calculate %$Prx3_{ox}$ for 17 normal tissue samples and 15 tumour samples. 9/17 (52.9%) samples were more oxidised in tumour than in normal tissue (Figure 4.11). There was no difference in mean %$Prx3_{ox}$ between normal (mean 68.8, SD 16.0) and tumour tissue (mean 72.0, SD 15.1) with a mean of difference 5.442, SEM of differences 3.265 (95% CI -1.418 – 12.30, p=0.1129). Standard deviations were larger for %$Prx3_{ox}$ than %$Prx2_{ox}$ showing greater variation in results.

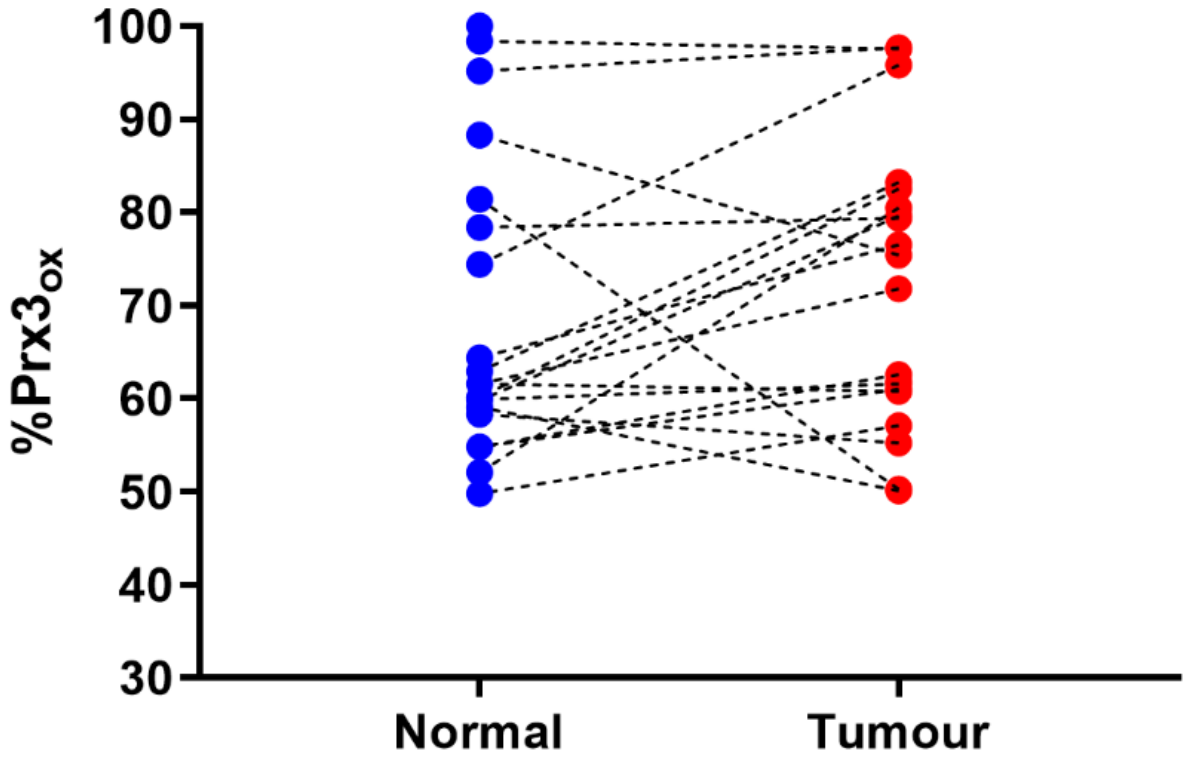

Figure 4.11. Relationship between peroxiredoxin 3 percentage oxidation for normal and tumour samples

Data was available for 22 normal and tumour biopsies (including two advanced adenomas, ID13 and ID21) for analysis using the protein carbonyl ELISA. There was no significant difference between mean carbonyls in normal tissue (mean 0.33 nmol/mg of protein, SD 0.21) compared with tumour tissue (0.31 nmol/mg of protein, SD 0.26) with a mean difference of 0.01973, SEM of differences 0.05337 (95% CI -0.1307 to 0.09127, p= 0.72). The results are shown in Figure 4.12.

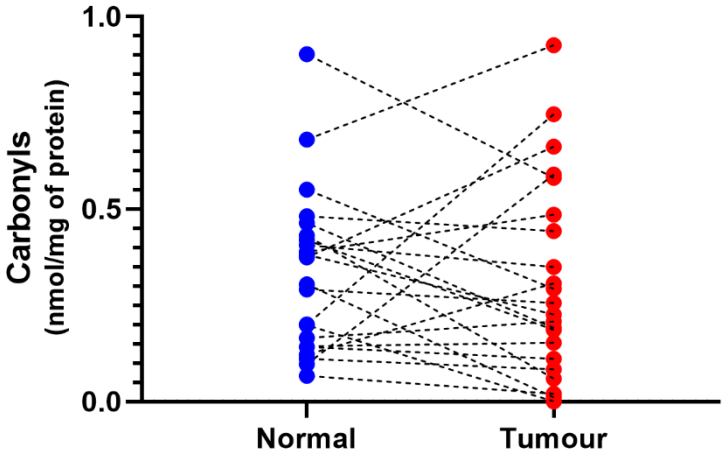

Figure 4.12. Relationship between protein carbonyl levels for normal and tumour samples

4.5.1.4 Comparison of Markers of Oxidative Stress for Responders and Non-responders

Due to low numbers of patients that underwent both nCRT and TME in the study period, pCR (n=2) and cCR (n=1) were combined as complete responders (CR). The outcome was also assessed by categorising patients by good response (GR) for TRG 0-1 or no GR for TRG 2-3. For the seven patients used to assess predictors the four biomarkers examined are presented in Table 4.5.

Table 4.5. Markers of oxidative stress as predictors of response after nCRT

Participant ID	%$Prx2_{ox}$		%$Prx3_{ox}$		Carbonyls (nmol/mg of protein)	
	Normal	**Tumour**	**Normal**	**Tumour**	**Normal**	**Tumour**
Complete Response						
1	74.0	62.1	78.4	79.4	0.200	0.746
10	69.4	68.7	64.4	76.5	0.406	0.350
11	67.5	67.0	NR	61.8	0.420	0.186
No Complete Response						
2	70.4	62.1	62.9	83.2	0.067	0.020
7	70.3	67.2	100.0	NR	0.680	0.925
14	65.4	71.0	74.4	95.8	0.464	0.191
16	64.6	87.2	54.8	62.6	0.481	0.443
Good Response (TRG 0-1)						
1	74.0	62.1	78.4	79.4	0.200	0.746
2	70.4	62.1	62.9	83.2	0.067	0.020
11	67.5	67.0	NR	61.8	0.420	0.186
16	64.6	87.2	54.8	62.6	0.481	0.443
No Good Response (TRG 2-3)						
7	70.3	67.2	100.0	NR	0.680	0.925
10	69.4	68.7	64.4	76.5	0.406	0.350
14	65.4	71.0	74.4	95.8	0.464	0.191

NR = no result

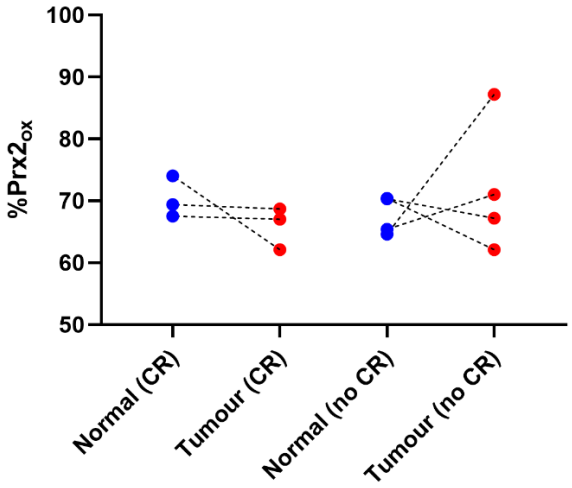

Figure 4.13. Peroxiredoxin 2 percentage oxidation in normal and tumour tissue for patients with and without complete response

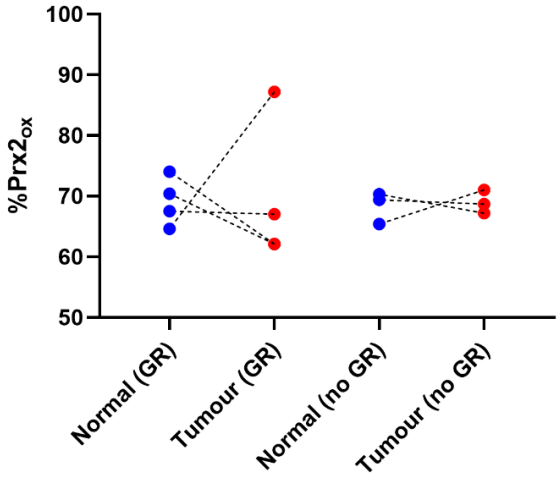

Figure 4.14. Peroxiredoxin 2 percentage oxidation in normal and tumour tissue for patients with and without good response

$\%Prx2_{ox}$ for CR compared with no CR and GR compared with no GR is shown in Figure 4.13 and Figure 4.14 respectively. There was no significant difference between normal and tumour samples when CR/no CR and GR/no GR were considered as outcomes. Participants in the CR group had lower $\%Prx2_{ox}$ in tumour tissue than normal tissue pre-treatment, although for two of the samples the difference was slight and the tumour and normal tissue were essentially equivocal.

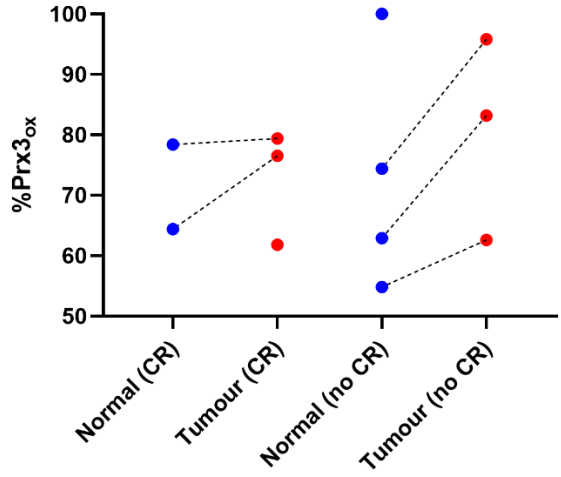

Figure 4.15. Peroxiredoxin 3 percentage oxidation in normal and tumour tissue for patients with and without complete response

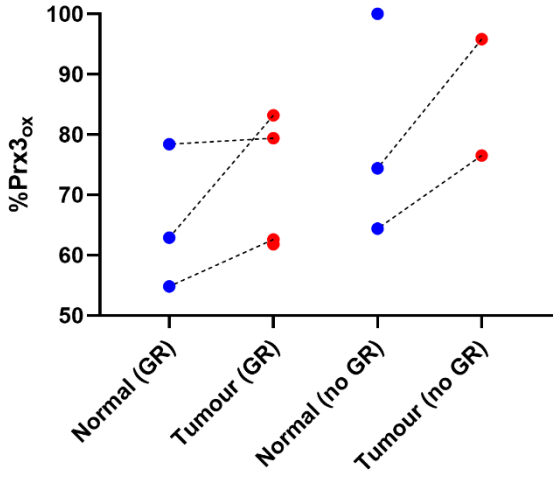

Figure 4.16. Peroxiredoxin 3 percentage oxidation in normal and tumour tissue for patients with and without good response

There was no significant difference between the $\%Prx3_{ox}$ of CR/no CR and GR/no GR (Figure 4.15 and Figure 4.16). All paired samples showed higher $\%Prx3_{ox}$ in tumour tissue than normal tissue.

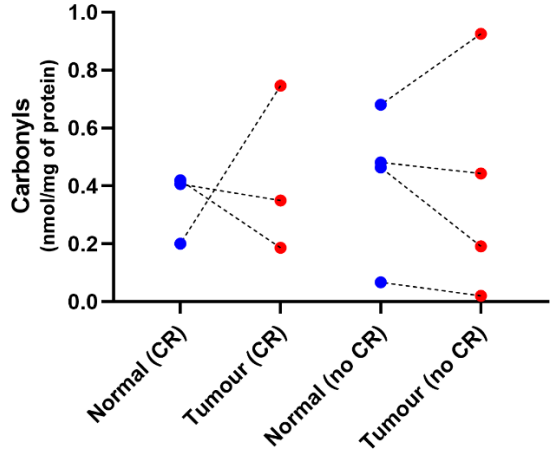

Figure 4.17. Protein carbonyl levels in normal and tumour tissue for patients with and without complete response

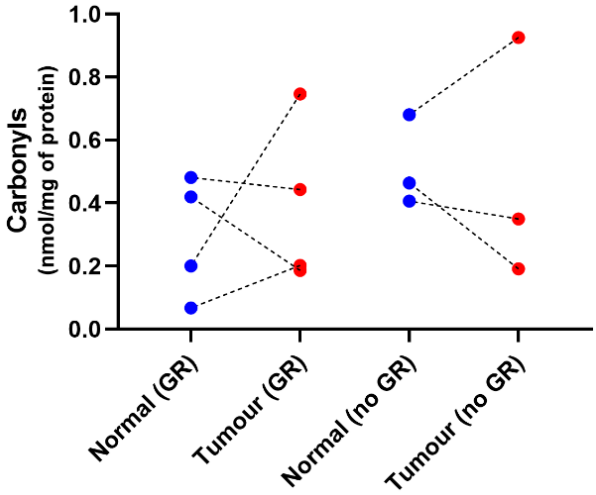

Figure 4.18. Protein carbonyl levels in normal and tumour tissue for patients with and without good response

There was no significant difference between the CR/no CR and GR/no GR groups for carbonyl level (Figure 4.17 and Figure 4.18). There was no trend in relative carbonyls between normal and tumour tissue.

4.5.1.5 Correlation Between Markers of Oxidative Stress

There was no significant correlation between markers of oxidative stress in tumour samples or normal biopsies when considered separately [%$Prx2_{ox}$ and %$Prx3_{ox}$ (normal r=0.1859, p=0.43; tumour r=0.06324, p=0.79), carbonyl and %$Prx2_{ox}$ (normal r=-0.1879, p=0.41; tumour r=0.1651, p=0.49)]. There was also no correlation when tumour and normal samples were combined (see Figure 4.19 and Figure 4.20). Only those participants with a valid result for both markers being assessed for correlation were included in this analysis.

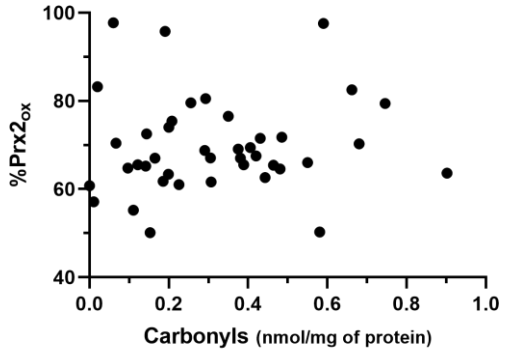

Figure 4.19. Peroxiredoxin 2 percentage oxidation vs. protein carbonyl level

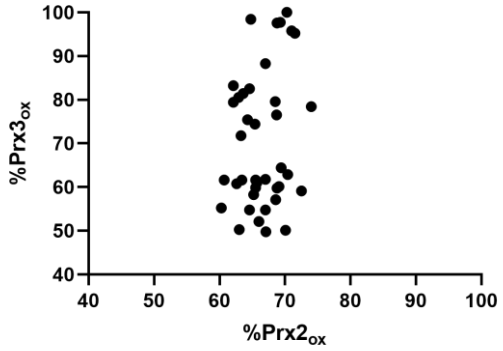

Figure 4.20. Peroxiredoxin 3 percentage oxidation vs. peroxiredoxin 2 percentage oxidation

4.5.1.6 Changes in Peroxiredoxin 2 Percentage Oxidation after Radiotherapy (CRT and SCRT)

5/7 normal tissue samples and 6/7 tumour tissue samples were found to have less $\%Prx2_{ox}$ in the resection specimen than in the pre-treatment sample. Mean $\%Prx2_{ox}$ was significantly lower for resection biopsies than pre-treatment biopsies for normal tissue on paired samples t test; the mean difference was 5.95 and SEM of differences 2.244 (95% CI 0.4585 -11.44, p=0.038) (Figure 4.21). There was also significantly less mean $\%Prx2_{ox}$ in tumour from resection specimens compared with pre-treatment biopsies, mean difference 4.312, SEM of differences 1.257 (95% CI 1.081 -7.542, p = 0.0186). The highest $\%Prx2_{ox}$ in the 'Resection Tumour' catgeory (red triangle in Figure 4.21) was a biopsy of mucosal scar in patient who had a pCR and was therefore excluded from statistical analysis.

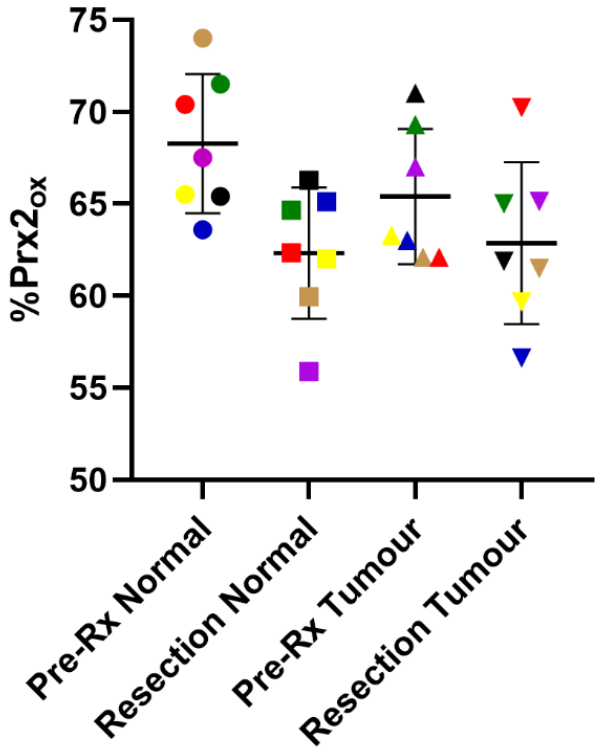

Figure 4.21. Variation in peroxiredoxin 2 percentage oxidation between pre-treatment and post-resection biopsies. Colours represent individial participants

4.6 Discussion

This study explored the use of oxidative stress biomarkers to predict the response of rectal cancer to radiotherapy. None of the markers that were examined have been investigated before in this setting, and there is no published literature assessing peroxiredoxin oxidation status in human tissue. This study has shown that this laboratory technique, most commonly performed in basic science and cell culture settings, can yield results in translational research. There are many more uncontrolled variables in obtaining tissue samples from human participants and the chasm between getting results from cell culture and real-life patient samples can be large. This study has also shown the potential to measure specific features of a complex functional biological system in translational research. Biomarkers that are focused on gene or protein presence or absence do not always consider that the activity of genes/proteins may not be a direct reflection of presence/quantification etc. i.e. activity is not always directly correlated with expression.

The primary aim of this pilot study was to ascertain whether the three biomarkers selected could act as predictive biomarkers of pathological response to radiotherapy for rectal

cancer. None of the markers investigated showed a significant association with pathological response. Although numbers were small overall, 2/7 participants achieved pCR which is higher than the expected pCR rate of 20% for this small group of participants. There was also an additional cCR, which although not identical to pCR, may act as a surrogate when participant numbers are low. This was a fortunate occurrence, because it allowed examination of the difference between responders and non-responders without large recruitment numbers overall. Only a marked difference is likely to have shown up in a study of this size, but a robust predictor needs to have high sensitivity and specificity, and it is reasonable to conclude that the markers of oxidative stress examined are unlikely to yield a clinically useful biomarker predictor using the methods described in this study.

Comparing markers of oxidative stress in tumour tissue and normal tissue, there was not a significant difference in absolute values for any marker. However when the relative Prx2 oxidation of tumour was compared with a paired normal sample there was generally less oxidation in tumour samples than in normal tissue. Tumours have long been stated to have higher levels of oxidative stress than normal tissue due to altered metabolic activity, transient hypoxia or infiltrating immune cells, although the evidence for this is relatively limited and based on cell culture studies (249). This raises the possibility that either there is not increased oxidant generation or there has been a concomitant increase in antioxidant defences. If anything Prx3 was more oxidised in tumour tissue, but difficulty with obtaining quality Prx3 blots limits interpretation of this. There was no difference in protein carbonyls when comparing normal and tumour tissue.

When $\%Prx2_{ox}$ was compared in pre-treatment and resection specimen samples, there was significantly more oxidation in pre-treatment than resection samples for both normal and tumour tissue. This was the opposite to expected, both because radiotherapy increases oxidative stress and because vascular ligation up to two hours before biopsy for the study was taken would also be expected to increase oxidation of peroxiredoxins. It may be that true oxidation in resection samples was even lower than what was measured but this is speculative.

The study found no correlation between markers of oxidative stress. This was not necessarily unexpected, as the mechanisms determining each marker are different and this is the reason

four different markers were chosen. It is difficult to draw any more meaningful conclusions from this.

There are a large number of limitations to this study. Study numbers overall were small, and the number of patients who underwent nCRT and TME was much lower due to attrition to alternative treatment strategies and treatment courses prolonged beyond the defined study period. Other limitations relate to uncontrolled variation in tissue sampling, tissue processing, western blotting and densitometry measurement.

Two advanced adenomas were included in the analysis which can be perceived as a weakness, but these results were in line with adenocarcinoma results and they were both large polyps with significant invasive potential. The inclusion of adenomas was not responsible for any significant finding.

Tumours are likely to have both intra and inter-tumoural variability in cell types. Oxidative stress is dynamic and will be affected by alterations to normal physiology including blood flow and inflammation. This could mean sampling the same tumour could yield different results based on location of the biopsy or fluctuating degrees of inflammation. Attempts were made to account for this by standardising tumour sampling as much as possible, but all tumours are unique and complete standardisation is not possible. Even normal tissue showed significant variation in the biomarkers examined, and normal tissue is likely to be more homogeneous than tumour tissue. Although we took normal tissue 5 cm from the tumour, it is possible there is a regional change in oxidative stress affecting both tumour and adjacent normal rectum due to an inflammatory response to the tumour.

Endoscopic biopsies are small, reported in the range of 3-10 mg (250). A small piece of tissue is generally more susceptible to oxidation with atmospheric exposure but a smaller piece of tissue will be penetrated more easily by NEM which will assist in trapping oxidation status of peroxiredoxins. Although predictable at the start of the study, it is impossible to say how this could have affected results.

Comparing density of band across different gels on different days with different conditions significantly impacts on reproducibility and comparability. By calculating the ratio of oxidised to reduced forms within a gel lane, some of this variability is negated as identical protein loading is assured for oxidised and reduced forms of each sample. Many of the densitometry

measurements could be reproduced with 10-20% variability which is usually regarded as satisfactory for an experimental method, $\%Prx3_{ox}$ being an exception to this.

We have been unable to measure total peroxiredoxin amounts with this method but this could be done by comparing with recombinant protein of known quantities. It is possible that more oxidation is occurring in some samples because there is less peroxiredoxin overall for that sample, so more of it is oxidised.

Western blotting is a technique that would have difficulties translating to use in clinical medicine due to the extensive time required, variability of results and difficulties with quantification. If peroxiredoxin oxidation was established as being of clinical utility, specific antibodies for the oxidised and reduced forms could be developed and used for immunohistochemical analysis in routine histopathological assessment.

This was a pilot study attempting to demonstrate the ability to assess the biomarkers of interest in human samples, and to look for any sign of associations with response in order to ascertain whether or not a larger scale study would be of benefit. It was a resource intensive study, and required a full-time researcher to identify patients, be constantly available to collect samples and process them immediately, quantify protein and manage sample storage. For a larger scale study the resources would be increased further and must be justified on the likelihood of a useful outcome. At this stage a larger scale replication of this study with the same methods and objectives is not justified.

5 Summary and Future Directions

This thesis has examined predictors of pathological response to chemoradiotherapy for rectal cancer. This was initially done by systematically reviewing the literature to ascertain the currently available evidence. The main findings from this systematic review were that clinical T and N stage, tumour size and CEA offer some predictive ability; they are routinely available and therefore their use is easily justified. Overall however, currently available predictors have very limited utility despite a vast amount of work on the subject. Subsequently a retrospective study was described in chapter 2 which identified clinicopathological predictors in 164 patients at Christchurch Hospital for the period 2013-2018. This supported previously reported findings of lower clinical nodal stage and smaller tumour size being associated with pCR. It also found BMI and anterior tumour position to be independent predictors of minimal-poor response by dichotomised TRG, results not previously reported. An attempt to create a clinical scoring system with genuine utility was unsuccessful based on the predictors identified in the study.

A literature review of the effect of ionising radiation on mitochondria and antioxidant systems in rectal cancer, with a specific focus on the peroxiredoxin family of antioxidants, formed chapter 3. This chapter outlined the theoretical basis for investigation of markers of oxidative stress, specifically peroxiredoxins, and the relevance these enzymes have to rectal cancer as potential predictors of radiosensitivity and targets for modulation of radiosensitivity.

Based on the background presented in chapter 3, chapter 4 reported a prospective pilot study investigating markers of oxidative stress as novel predictive biomarkers of the response to radiotherapy for rectal cancer. Peroxiredoxin oxidation status and protein carbonyls have been shown in previous studies to be biomarkers of oxidative stress in human cells. Although this study established the viability of laboratory techniques quantifying percentage oxidation of peroxiredoxins that has not been reported before in translational research, it failed to identify a significant predictor of response. Peroxiredoxin 2, peroxiredoxin 3 and protein carbonyls were not found to have any predictive ability for the pathological response to radiotherapy for rectal cancer. It is a long-held belief that tumour cells are under increased oxidative stress, and radiotherapy is known to induce massive oxidative stress in cells. It is likely that oxidative stress plays a major role in tumour

cell death by radiotherapy, but redox homeostasis is a complex system. A marker of balance in one antioxidant system may not be a clinically useful marker because compensatory antioxidant systems may be upregulated. Tumour heterogeneity is a significant issue that can detract from reproducibility of laboratory techniques such as those used in chapter 4. Tissue samples from one area of tumour might vary significantly from elsewhere, and absolute consistency of sampling is impossible as all tumours are unique. This is a major limitation of the experimental work performed and a challenge to overcome in future work in the field. Markers of oxidative stress remain potential predictors of pathological response to radiotherapy for rectal cancer, but alternative avenues need to be explored. The role of oxidative stress in carcinogenesis is a developing field, and there remains much that is unknown about the role of oxidative stress in human colorectal carcinogenesis (251). As the exact redox mechanisms leading to colorectal cancer development and progression are elucidated in humans, the most promising lines of investigation should become apparent with regard to radiosensitivity of rectal cancer. It is however likely that a complete understanding of redox homeostasis will not be achieved for some time, and an inability to account for a large number of factors in vivo may even prevent this approach being introduced to clinical practice.

This thesis has focused on identification of predictors of response to radiotherapy for rectal cancer, but the ability to manipulate tumour biology to promote cancer cell death is the logical next step if a modifiable aspect of a biological system was found to influence response to therapy. It is possible to induce oxidative stress in laboratory models through the addition of reactive oxidants, and the ability to induce oxidative stress in tumour cells in vivo could increase radiosensitivity or even provide a mode of tumour cell death as a stand-alone treatment. This work is currently far from clinical practice but there is significant potential for future developments in this area. A strategy to increase radiosensitivity which has been explored for cervical and head and neck squamous cell carcinoma is hyperbaric oxygen delivery (252). The evidence for efficacy is limited, and logistical difficulties in delivering hyperbaric oxygen in close temporal association with radiotherapy limits the use of this, as well as the potential for increased side effects from the effect on irradiated normal tissue. These limitations could possibly be overcome if techniques to increase oxygen tension could be targeted to tumour tissue.

The strengths of this thesis are that a global view of predictors of response to radiotherapy has been taken, considering clinical, biomarker and radiological predictors. A comprehensive review of a large amount of literature provided context and justification for the original research in the chapters that followed. Chapter 4 reported a completely novel approach to biomarker predictors of the response to radiotherapy for rectal cancer. Specific weaknesses have been discussed in each chapter.

Rectal cancer is a heterogeneous disease. Molecular sub-types of colorectal cancer were decided by consensus in 2015 (253) but have yet to be externally validated. It is possible that molecular features have some impact on sensitivity to radiotherapy for rectal cancer as they are known to impact responsiveness to medical treatment for colorectal cancer (254-257), and may provide an avenue for biomarker based prediction in the future. The way forward is unlikely to be based on any single category of predictor, but is likely to incorporate the strongest predictors from the clinicopathological, biomarker and radiological predictor categories to create a composite scoring system. This is difficult to apply and requires large patient cohorts both to develop and validate. It is likely to incorporate clinicopathological factors already known (e.g. cT and cN stage, tumour size, CEA), and may incorporate novel biomarkers and advanced imaging features that have yet to be elucidated.

Predicting response to treatment is an important aspect of advancing treatment for rectal cancer. In a developing era of personalised medicine (258), targeting treatment to a patient based on characteristics of their disease is likely to be the way forward. However it is achieved, personalising medicine to avoid delivering morbid treatments to large numbers of patients while only a modest proportion of people benefit must be a goal for all clinicians, especially those treating cancer. In the treatment of rectal cancer the morbidity and mortality of surgery and radiotherapy is particularly apparent.

Rectal cancer has seen major advances in treatment since total mesorectal excision was popularised in the 1980s and 1990s and the use of radiotherapy became routine; surgical innovation continues with laparoscopic, robotic and transanal total mesorectal excision approaches aiming to improve outcomes for our patients. As great as surgical progress is, often the best outcome for the patient is achieved when we can avoid the need to operate at all. While we must not compromise oncological safety in the pursuit of non-operative management, watch-and-wait treatment appears to be a viable alternative that is edging

towards mainstream practice (9). The question will be how do we select patients most appropriately, and how can we increase the number of patients able to safely receive watch-and-wait management? Predicting the response to radiotherapy for rectal cancer could be a key piece of the puzzle.

Appendix A

Table A1. Tissue biomarkers investigated in only one study assessed in the systematic review.

Biomarker	Ref
XRCC1	(95)
PARP1	(95)
FGF4	(260)
FGF2	(46)
PLK1	(261)
VRK1/ VRK2	(62)
PIGF	(104)
SDF-1α	(104)
TCF4	(72)
GHRH-R	(265)
Hsp90	(265)
FAK	(268)
FN1	(270)
COL3A1	(270)
CD34	(64)
CA9	(64)
DNAJC12	(273)
REG4	(274)
PLA2G2A	(276)
HMGB1	(277)
mlh-1	(73)
msh-2	(73)
Ku70	(73)
SMAD3	(45)
TGFβ	(45)
HSD17B2	(278)

Biomarker	Ref
CPS1	(259)
CA-IX	(105)
LDH5	(106)
PDK1	(106)
Asparagine synthetase	(262)
Rsf-1	(263)
Thymidine phosphorylase	(107)
ABCC4	(39)
ANXA1	(264)
LC3β /autophagy related proteins	(266)
CD133	(267)
SMAC	(269)
NK-KB	(271)
GRP78	(272)
Cripto-1	(272)
GOLPH3	(69)
CXCR4	(64)
YKL-40	(275)
c-Met	(275)
ERCC1	(116)
MTHFR	(116)
DPYD	(116)
CA-IX	(105)
Beta catenin	(96)
p27	(101)
HMGCS2	(278)

Bibliography

1. Ministry of Health. Cancer: Historical summary 1948–2015 Wellington: Ministry of Health2018 [Web page]. Available from: https://www.health.govt.nz/publication/cancer-historical-summary-1948-2015.
2. Holme O, Bretthauer M, Fretheim A, Odgaard-Jensen J, Hoff G. Flexible sigmoidoscopy versus faecal occult blood testing for colorectal cancer screening in asymptomatic individuals. Cochrane Database Syst Rev. 2013(9):Cd009259.
3. Gandhi J, Davidson C, Hall C, Pearson J, Eglinton T, Wakeman C, et al. Population-based study demonstrating an increase in colorectal cancer in young patients. Br J Surg. 2017;104(8):1063-8.
4. Heriot A, Platell C, Byrne C, Chapuis P, Doudle M, McMurrick P, et al. The Bi-National Colorectal Cancer Audit Report 2017. 2017.
5. Keane C, Wells C, O'Grady G, Bissett IP. Defining low anterior resection syndrome: a systematic review of the literature. Colorectal Dis. 2017;19(8):713-22.
6. Pilipshen SJ, Heilweil M, Quan SH, Sternberg SS, Enker WE. Patterns of pelvic recurrence following definitive resections of rectal cancer. Cancer. 1984;53(6):1354-62.
7. Heald RJ, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? Br J Surg. 1982;69(10):613-6.
8. Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med. 2001;345(9):638-46.
9. Tekkis P, Tait D, Cunningham D, Brown G. Is organ preservation in rectal cancer ready for prime time? The Lancet. 2018;391(10139):2480-2.
10. Lombardi L, Gebbia V, Silvestris N, Testa A, Colucci G, Maiello E. Adjuvant therapy in colon cancer. Oncology. 2009;77 Suppl 1:50-6.
11. Bosset J-F, Collette L, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, et al. Chemotherapy with Preoperative Radiotherapy in Rectal Cancer. 2006;355(11):1114-23.
12. Bujko K, Nowacki MP, Nasierowska-Guttmejer A, Michalski W, Bebenek M, Kryj M. Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer. Br J Surg. 2006;93(10):1215-23.
13. McCarthy K, Pearson K, Fulton R, Hewitt J. Pre-operative chemoradiation for non-metastatic locally advanced rectal cancer. Cochrane Database Syst Rev. 2012;12:Cd008368.
14. O'Connell MJ, Colangelo LH, Beart RW, Petrelli NJ, Allegra CJ, Sharif S, et al. Capecitabine and Oxaliplatin in the Preoperative Multimodality Treatment of Rectal Cancer: Surgical End Points From National Surgical Adjuvant Breast and Bowel Project Trial R-04. Journal of Clinical Oncology. 2014;32(18):1927-34.
15. Macchia G, Gambacorta MA, Masciocchi C, Chiloiro G, Mantello G, di Benedetto M, et al. Time to surgery and pathologic complete response after neoadjuvant chemoradiation in rectal cancer: A population study on 2094 patients. Clinical and translational radiation oncology. 2017;4:8-14.
16. Ceelen W, Boterberg T, Pattyn P, van Eijkeren M, Gillardin JM, Demetter P, et al. Neoadjuvant chemoradiation versus hyperfractionated accelerated radiotherapy in locally advanced rectal cancer. Annals of surgical oncology. 2007;14(2):424-31.
17. Ngan SY, Burmeister B, Fisher RJ, Solomon M, Goldstein D, Joseph D, et al. Randomized trial of short-course radiotherapy versus long-course chemoradiation comparing rates of local recurrence in patients with T3 rectal cancer: Trans-Tasman Radiation Oncology Group trial 01.04. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(31):3827-33.
18. Sebag-Montefiore D. Radiotherapy for rectal cancer: Short course versus long course - When and how. EJC supplements : EJC : official journal of EORTC, European Organization for Research and Treatment of Cancer [et al]. 2013;11(2):282-3.

19. Pach R, Kulig J, Richter P, Gach T, Szura M, Kowalska T. Randomized clinical trial on preoperative radiotherapy 25 Gy in rectal cancer--treatment results at 5-year follow-up. Langenbeck's archives of surgery. 2012;397(5):801-7.
20. Francois Y, Nemoz CJ, Baulieux J, Vignal J, Grandjean JP, Partensky C, et al. Influence of the interval between preoperative radiation therapy and surgery on downstaging and on the rate of sphincter-sparing surgery for rectal cancer: the Lyon R90-01 randomized trial. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 1999;17(8):2396.
21. Erlandsson J, Holm T, Pettersson D, Berglund A, Cedermark B, Radu C, et al. Optimal fractionation of preoperative radiotherapy and timing to surgery for rectal cancer (Stockholm III): a multicentre, randomised, non-blinded, phase 3, non-inferiority trial. The Lancet Oncology. 2017;18(3):336-46.
22. Song JH, Jeong JU, Lee JH, Kim SH, Cho HM, Um JW, et al. Preoperative chemoradiotherapy versus postoperative chemoradiotherapy for stage II-III resectable rectal cancer: a meta-analysis of randomized controlled trials. Radiation oncology journal. 2017;35(3):198-207.
23. Sauer R, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, et al. Preoperative versus Postoperative Chemoradiotherapy for Rectal Cancer. New England Journal of Medicine. 2004;351(17):1731-40.
24. Sebag-Montefiore D, Stephens RJ, Steele R, Monson J, Grieve R, Khanna S, et al. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. Lancet (London, England). 2009;373(9666):811-20.
25. Birgisson H, Pahlman L, Gunnarsson U, Glimelius B. Late adverse effects of radiation therapy for rectal cancer - a systematic overview. Acta oncologica (Stockholm, Sweden). 2007;46(4):504-16.
26. Ryan JE, Warrier SK, Lynch AC, Ramsay RG, Phillips WA, Heriot AG. Predicting pathological complete response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer: a systematic review. Colorectal Dis. 2016;18(3):234-46.
27. Kong J, Guerra G, K Warrier S, Lynch A, Michael M, Y Ngan S, et al. Prognostic Value of Tumour Regression Grade in Locally Advanced Rectal Cancer: A Systematic Review and Meta-analysis2018.
28. Kim SH, Chang HJ, Kim DY, Park JW, Baek JY, Kim SY, et al. What Is the Ideal Tumor Regression Grading System in Rectal Cancer Patients after Preoperative Chemoradiotherapy? Cancer research and treatment : official journal of Korean Cancer Association. 2016;48(3):998-1009.
29. Fokas E, Strobel P, Fietkau R, Ghadimi M, Liersch T, Grabenbauer GG, et al. Tumor Regression Grading After Preoperative Chemoradiotherapy as a Prognostic Factor and Individual-Level Surrogate for Disease-Free Survival in Rectal Cancer. J Natl Cancer Inst. 2017;109(12).
30. Habr-Gama A, Perez RO, Nadalin W, Sabbaga J, Ribeiro U, Jr., Silva e Sousa AH, Jr., et al. Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. Annals of surgery. 2004;240(4):711-8.
31. van der Valk MJM, Hilling DE, Bastiaannet E, Meershoek-Klein Kranenbarg E, Beets GL, Figueiredo NL, et al. Long-term outcomes of clinical complete responders after neoadjuvant treatment for rectal cancer in the International Watch & Wait Database (IWWD): an international multicentre registry study. The Lancet. 2018;391(10139):2537-45.
32. Renehan AG, Malcomson L, Emsley R, Gollins S, Maw A, Myint AS, et al. Watch-and-wait approach versus surgical resection after chemoradiotherapy for patients with rectal cancer (the OnCoRe project): a propensity-score matched cohort analysis. The Lancet Oncology. 2016;17(2):174-83.
33. Garcia-Aguilar J, Chow OS, Smith DD, Marcet JE, Cataldo PA, Varma MG, et al. Effect of adding mFOLFOX6 after neoadjuvant chemoradiation in locally advanced rectal cancer: a multicentre, phase 2 trial. The Lancet Oncology. 2015;16(8):957-66.
34. Probst CP, Becerra AZ, Aquina CT, Tejani MA, Hensley BJ, Gonzalez MG, et al. Watch and Wait?--Elevated Pretreatment CEA Is Associated with Decreased Pathological Complete Response in Rectal Cancer. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2016;20(1):43-52; discussion

35. Yu J, Xu Q, Song JC, Li Y, Dai X, Huang DY, et al. The value of diffusion kurtosis magnetic resonance imaging for assessing treatment response of neoadjuvant chemoradiotherapy in locally advanced rectal cancer. European radiology. 2017;27(5):1848-57.
36. Bang JI, Ha S, Kang SB, Lee KW, Lee HS, Kim JS, et al. Prediction of neoadjuvant radiation chemotherapy response and survival using pretreatment [(18)F]FDG PET/CT scans in locally advanced rectal cancer. European journal of nuclear medicine and molecular imaging. 2016;43(3):422-31.
37. Armstrong D, Raissouni S, Price Hiller J, Mercer J, Powell E, MacLean A, et al. Predictors of Pathologic Complete Response After Neoadjuvant Treatment for Rectal Cancer: A Multicenter Study. Clinical colorectal cancer. 2015;14(4):291-5.
38. Kalady MF, de Campos-Lobato LF, Stocchi L, Geisler DP, Dietz D, Lavery IC, et al. Predictive factors of pathologic complete response after neoadjuvant chemoradiation for rectal cancer. Annals of surgery. 2009;250(4):582-9.
39. Yu ZQ, Zhang C, Wang H, Lao XY, Chai R, Gao XH, et al. Downregulation of ATP-binding cassette subfamily C member 4 increases sensitivity to neoadjuvant radiotherapy for locally advanced rectal carcinoma. Dis Colon Rectum. 2013;56(5):600-8.
40. Wallin U, Rothenberger D, Lowry A, Luepker R, Mellgren A. CEA - a predictor for pathologic complete response after neoadjuvant therapy for rectal cancer. Diseases of the colon and rectum. 2013;56(7):859-68.
41. Moureau-Zabotto L, Farnault B, de Chaisemartin C, Esterni B, Lelong B, Viret F, et al. Predictive factors of tumor response after neoadjuvant chemoradiation for locally advanced rectal cancer. International journal of radiation oncology, biology, physics. 2011;80(2):483-91.
42. Park CH, Kim HC, Cho YB, Yun SH, Lee WY, Park YS, et al. Predicting tumor response after preoperative chemoradiation using clinical parameters in rectal cancer. World J Gastroenterol. 2011;17(48):5310-6.
43. Abdul-Jalil KI, Sheehan KM, Toomey S, Schmid J, Prehn J, O'Grady A, et al. The frequencies and clinical implications of mutations in 33 kinase-related genes in locally advanced rectal cancer: a pilot study. Annals of surgical oncology. 2014;21(8):2642-9.
44. Dreyer SB, Powell AGMT, McSorley ST, Waterston A, Going JJ, Edwards J, et al. The Pretreatment Systemic Inflammatory Response is an Important Determinant of Poor Pathologic Response for Patients Undergoing Neoadjuvant Therapy for Rectal Cancer. Annals of Surgical Oncology. 2017;24(5):1295-303.
45. Huang MY, Lin CH, Huang CM, Tsai HL, Huang CW, Yeh YS, et al. Relationships between SMAD3 expression and preoperative fluoropyrimidine-based chemoradiotherapy response in locally advanced rectal cancer patients. World journal of surgery. 2015;39(5):1257-67.
46. Li CF, He HL, Wang JY, Huang HY, Wu TF, Hsing CH, et al. Fibroblast growth factor receptor 2 overexpression is predictive of poor prognosis in rectal cancer patients receiving neoadjuvant chemoradiotherapy. Journal of clinical pathology. 2014;67(12):1056-61.
47. Tada N, Kawai K, Tsuno NH, Ishihara S, Yamaguchi H, Sunami E, et al. Prediction of the preoperative chemoradiotherapy response for rectal cancer by peripheral blood lymphocyte subsets. World journal of surgical oncology. 2015;13:30.
48. Joye I, Debucquoy A, Fieuws S, Wolthuis A, Sagaert X, D'Hoore A, et al. Can clinical factors be used as a selection tool for an organ-preserving strategy in rectal cancer? Acta oncologica (Stockholm, Sweden). 2016;55(8):1047-52.
49. Lorimer PD, Motz BM, Kirks RC, Boselli DM, Walsh KK, Prabhu RS, et al. Pathologic Complete Response Rates After Neoadjuvant Treatment in Rectal Cancer: An Analysis of the National Cancer Database. Annals of surgical oncology. 2017;24(8):2095-103.
50. Caudle AS, Kim HJ, Tepper JE, O'Neil BH, Lange LA, Goldberg RM, et al. Diabetes mellitus affects response to neoadjuvant chemoradiotherapy in the management of rectal cancer. Annals of surgical oncology. 2008;15(7):1931-6.
51. Huh JW, Kim HR, Kim YJ. Clinical prediction of pathological complete response after preoperative chemoradiotherapy for rectal cancer. Diseases of the colon and rectum. 2013;56(6):698-703.

52. Sun Y, Xu Z, Lin H, Lu X, Huang Y, Huang S, et al. Impact of body mass index on treatment outcome of neoadjuvant chemoradiotherapy in locally advanced rectal cancer. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2017;43(10):1828-34.
53. Al-Sukhni E, Attwood K, Mattson DM, Gabriel E, Nurkin SJ. Predictors of Pathologic Complete Response Following Neoadjuvant Chemoradiotherapy for Rectal Cancer. Annals of surgical oncology. 2016;23(4):1177-86.
54. Qiu HZ, Wu B, Xiao Y, Lin GL. Combination of differentiation and T stage can predict unresponsiveness to neoadjuvant therapy for rectal cancer. Colorectal Dis. 2011;13(12):1353-60.
55. McCawley N, Clancy C, O'Neill BD, Deasy J, McNamara DA, Burke JP. Mucinous Rectal Adenocarcinoma Is Associated with a Poor Response to Neoadjuvant Chemoradiotherapy: A Systematic Review and Meta-analysis. Diseases of the colon and rectum. 2016;59(12):1200-8.
56. Reggiani Bonetti L, Lionti S, Domati F, Barresi V. Do pathological variables have prognostic significance in rectal adenocarcinoma treated with neoadjuvant chemoradiotherapy and surgery? World journal of gastroenterology. 2017;23(8):1412-23.
57. Jayanand SB, Seshadri RA, Tapkire R. Signet ring cell histology and non-circumferential tumors predict pathological complete response following neoadjuvant chemoradiation in rectal cancers. International journal of colorectal disease. 2011;26(1):23-7.
58. Rogers AC, Gibbons D, Hanly AM, Hyland JM, O'Connell PR, Winter DC, et al. Prognostic significance of tumor budding in rectal cancer biopsies before neoadjuvant therapy. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2014;27(1):156-62.
59. Li C, Wang Z, Liu F, Zhu J, Yang L, Cai G, et al. CXCL10 mRNA expression predicts response to neoadjuvant chemoradiotherapy in rectal cancer patients. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35(10):9683-91.
60. Lee JH, Kim SH, Kim JG, Cho HM, Shim BY. Preoperative chemoradiotherapy (CRT) followed by laparoscopic surgery for rectal cancer: predictors of the tumor response and the long-term oncologic outcomes. Int J Radiat Oncol Biol Phys. 2011;81(2):431-8.
61. Committee obotEG, Glynne-Jones R, Wyrwicz L, Tiret E, Brown G, Rödel C, et al. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Ann Oncol. 2017;28(suppl_4):iv22-iv40.
62. Del Puerto-Nevado L, Marin-Arango JP, Fernandez-Acenero MJ, Arroyo-Manzano D, Martinez-Useros J, Borrero-Palacios A, et al. Predictive value of vrk 1 and 2 for rectal adenocarcinoma response to neoadjuvant chemoradiation therapy: a retrospective observational cohort study. BMC Cancer. 2016;16:519.
63. De Felice F, Izzo L, Musio D, Magnante AL, Bulzonetti N, Pugliese F, et al. Clinical predictive factors of pathologic complete response in locally advanced rectal cancer. Oncotarget. 2016;7(22):33374-80.
64. Guedj N, Bretagnol F, Rautou PE, Deschamps L, Cazals-Hatem D, Bedossa P, et al. Predictors of tumor response after preoperative chemoradiotherapy for rectal adenocarcinomas. Human pathology. 2011;42(11):1702-9.
65. Yan H, Wang R, Zhu K, Zhao W, Jiang S, Feng R, et al. Predictors of sensitivity to preoperative chemoradiotherapy of rectal adenocarcinoma. Tumori. 2011;97(6):717-23.
66. Patel SV, Roxburgh CS, Vakiani E, Shia J, Smith JJ, Temple LK, et al. Distance to the anal verge is associated with pathologic complete response to neoadjuvant therapy in locally advanced rectal cancer. Journal of surgical oncology. 2016;114(5):637-41.
67. Restivo A, Zorcolo L, Cocco IM, Manunza R, Margiani C, Marongiu L, et al. Elevated CEA levels and low distance of the tumor from the anal verge are predictors of incomplete response to chemoradiation in patients with rectal cancer. Annals of surgical oncology. 2013;20(3):864-71.
68. Garland ML, Vather R, Bunkley N, Pearse M, Bissett IP. Clinical tumour size and nodal status predict pathologic complete response following neoadjuvant chemoradiotherapy for rectal cancer. International journal of colorectal disease. 2014;29(3):301-7.

69. Zhu K, Zhao Q, Yue J, Shi P, Yan H, Xu X, et al. GOLPH3 overexpression correlates with poor response to neoadjuvant therapy and prognosis in locally advanced rectal cancer. Oncotarget. 2016;7(42):68328-38.
70. Yeo SG, Kim DY, Chang HJ, Park JW, Oh JH, Kim BC, et al. Reappraisal of pretreatment carcinoembryonic antigen in patients with rectal cancer receiving preoperative chemoradiotherapy. Tumori. 2013;99(1):93-9.
71. Hsu CY, Wang CW, Kuo CC, Chen YH, Lan KH, Cheng AL, et al. Tumor compactness improves the preoperative volumetry-based prediction of the pathological complete response of rectal cancer after preoperative concurrent chemoradiotherapy. Oncotarget. 2017;8(5):7921-34.
72. Dou X, Wang R, Meng X, Yan H, Jiang S, Zhu K, et al. The prognostic role of TCF4 expression in locally advanced rectal cancer patients treated with neoadjuvant chemoradiotherapy. Cancer biomarkers : section A of Disease markers. 2015;15(2):181-8.
73. Hur H, Kim NK, Min BS, Baik SH, Lee KY, Koom WS, et al. Can a biomarker-based scoring system predict pathologic complete response after preoperative chemoradiotherapy for rectal cancer? Dis Colon Rectum. 2014;57(5):592-601.
74. Colloca G, Venturino A, Vitucci P. Pre-treatment carcinoembryonic antigen and outcome of patients with rectal cancer receiving neo-adjuvant chemo-radiation and surgical resection: a systematic review and meta-analysis. Medical oncology (Northwood, London, England). 2017;34(10):177.
75. Lee JH, Hyun JH, Kim DY, Yoo BC, Park JW, Kim SY, et al. The role of fibrinogen as a predictor in preoperative chemoradiation for rectal cancer. Annals of surgical oncology. 2015;22(1):209-15.
76. Li QW, Zheng RL, Ling YH, Wang QX, Xiao WW, Zeng ZF, et al. Prediction of tumor response after neoadjuvant chemoradiotherapy in rectal cancer using (18)fluorine-2-deoxy-D-glucose positron emission tomography-computed tomography and serum carcinoembryonic antigen: a prospective study. Abdominal radiology (New York). 2016;41(8):1448-55.
77. Clarke TL, White DA, Osborne ME, Shaw AM, Smart NJ, Daniels IR. Predicting response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer with serum biomarkers. Annals of the Royal College of Surgeons of England. 2017;99(5):373-7.
78. Moreno Garcia V, Cejas P, Blanco Codesido M, Feliu Batlle J, de Castro Carpeno J, Belda-Iniesta C, et al. Prognostic value of carcinoembryonic antigen level in rectal cancer treated with neoadjuvant chemoradiotherapy. International journal of colorectal disease. 2009;24(7):741-8.
79. Park JW, Lim SB, Kim DY, Jung KH, Hong YS, Chang HJ, et al. Carcinoembryonic antigen as a predictor of pathologic response and a prognostic factor in locally advanced rectal cancer patients treated with preoperative chemoradiotherapy and surgery. Int J Radiat Oncol Biol Phys. 2009;74(3):810-7.
80. Andrade VA, Coy CS, Leal RF, Fagundes JJ, Martinez CA, Ayrizono Mde L. NEOADJUVANT THERAPY AND SURGERY FOR RECTAL CANCER. Comparative study between partial and complete pathological response. Arq Gastroenterol. 2016;53(3):163-8.
81. Kim HJ, Choi GS, Park JS, Park S, Kawai K, Watanabe T. Clinical significance of thrombocytosis before preoperative chemoradiotherapy in rectal cancer: predicting pathologic tumor response and oncologic outcome. Annals of surgical oncology. 2015;22(2):513-9.
82. Dou X, Wang RB, Yan HJ, Jiang SM, Meng XJ, Zhu KL, et al. Circulating lymphocytes as predictors of sensitivity to preoperative chemoradiotherapy in rectal cancer cases. Asian Pacific journal of cancer prevention : APJCP. 2013;14(6):3881-5.
83. Hodek M, Sirak I, Ferko A, Orhalmi J, Hovorkova E, Hadzi Nikolov D, et al. Neoadjuvant chemoradiotherapy of rectal carcinoma : Baseline hematologic parameters influencing outcomes. Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al]. 2016;192(9):632-40.
84. Lino-Silva LS, Salcedo-Hernandez RA, Ruiz-Garcia EB, Garcia-Perez L, Herrera-Gomez A. Pre-operative Neutrophils/Lymphocyte Ratio in Rectal Cancer Patients with Preoperative Chemoradiotherapy. Medical archives (Sarajevo, Bosnia and Herzegovina). 2016;70(4):256-60.

85. Shen L, Zhang H, Liang L, Li G, Fan M, Wu Y, et al. Baseline neutrophil-lymphocyte ratio (>/=2.8) as a prognostic factor for patients with locally advanced rectal cancer undergoing neoadjuvant chemoradiation. Radiation oncology (London, England). 2014;9:295.
86. Sun W, Li G, Wan J, Zhu J, Shen W, Zhang Z. Circulating tumor cells: A promising marker of predicting tumor response in rectal cancer patients receiving neoadjuvant chemo-radiation therapy. Oncotarget. 2016;7(43):69507-17.
87. Khan AA, Klonizakis M, Shabaan A, Glynne-Jones R. Association between pretreatment haemoglobin levels and morphometric characteristics of the tumour, response to neoadjuvant treatment and long-term outcomes in patients with locally advanced rectal cancers. Colorectal Dis. 2013;15(10):1232-7.
88. Matsutani S, Shibutani M, Maeda K, Nagahara H, Fukuoka T, Nakao S, et al. Significance of tumor-infiltrating lymphocytes before and after neoadjuvant therapy for rectal cancer. Cancer science. 2018;109(4):966-79.
89. McCoy MJ, Hemmings C, Anyaegbu CC, Austin SJ, Lee-Pullen TF, Miller TJ, et al. Tumour-infiltrating regulatory T cell density before neoadjuvant chemoradiotherapy for rectal cancer does not predict treatment response. Oncotarget. 2017;8(12):19803-13.
90. McCoy MJ, Hemmings C, Miller TJ, Austin SJ, Bulsara MK, Zeps N, et al. Low stromal Foxp3+ regulatory T-cell density is associated with complete response to neoadjuvant chemoradiotherapy in rectal cancer. British journal of cancer. 2015;113(12):1677-86.
91. Teng F, Meng X, Kong L, Mu D, Zhu H, Liu S, et al. Tumor-infiltrating lymphocytes, forkhead box P3, programmed death ligand-1, and cytotoxic T lymphocyte-associated antigen-4 expressions before and after neoadjuvant chemoradiation in rectal cancer. Translational research : the journal of laboratory and clinical medicine. 2015;166(6):721-32.e1.
92. Fedchenko N, Reifenrath J. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review. Diagnostic pathology. 2014;9:221.
93. Kikuchi M, Mikami T, Sato T, Tokuyama W, Araki K, Watanabe M, et al. High Ki67, Bax, and thymidylate synthase expression well correlates with response to chemoradiation therapy in locally advanced rectal cancers: proposal of a logistic model for prediction. British journal of cancer. 2009;101(1):116-23.
94. Jafarian AH, Kermani AT, Esmaeili J, Roshan NM, Seilanian-Toosi M, Omidi AA, et al. The role of COX-2 and Ki-67 over-expression in the prediction of pathologic response of rectal cancer to neoadjuvant chemoradiation therapy. Indian journal of cancer. 2016;53(4):548-51.
95. Carlomagno C, Pepe S, D'Armiento FP, D'Armiento M, Cannella L, De Stefano A, et al. Predictive factors of complete response to neoadjuvant chemoradiotherapy in patients with rectal cancer. Oncology. 2010;78(5-6):369-75.
96. Garcia-Florez LJ, Gomez-Alvarez G, Frunza AM, Barneo-Serra L, Martinez-Alonso C, Fresno-Forcelledo MF. Predictive markers of response to neoadjuvant therapy in rectal cancer. The Journal of surgical research. 2015;194(1):120-6.
97. Suzuki T, Sadahiro S, Tanaka A, Okada K, Saito G, Kamijo A, et al. Predictive markers of chemoradiotherapy for rectal cancer: comparison of biopsy specimens taken before and about 1 week after the start of chemoradiotherapy. International journal of clinical oncology. 2015;20(6):1130-9.
98. Havelund BM, Sorensen FB, Ploen J, Lindebjerg J, Spindler KLG, Jakobsen A. Immunohistological expression of HIF-1alpha, GLUT-1, Bcl-2 and Ki-67 in consecutive biopsies during chemoradiotherapy in patients with rectal cancer. Apmis. 2013;121(2):127-38.
99. Terzi C, Canda AE, Sagol O, Atila K, Sonmez D, Fuzun M, et al. Survivin, p53, and Ki-67 as predictors of histopathologic response in locally advanced rectal cancer treated with preoperative chemoradiotherapy. International journal of colorectal disease. 2008;23(1):37-45.
100. Sim SH, Kang MH, Kim YJ, Lee KW, Kim DW, Kang SB, et al. P21 and CD166 as predictive markers of poor response and outcome after fluorouracil-based chemoradiotherapy for the patients with rectal cancer. BMC Cancer. 2014;14:241.

101. Edden Y, Wexner SD, Berho M. The use of molecular markers as a method to predict the response to neoadjuvant therapy for advanced stage rectal adenocarcinoma. Colorectal Dis. 2012;14(5):555-61.
102. Peng H, You K, Zhang R, Xi S, Zhang T, Dong J, et al. Predictive value of APAF-1 and COX-2 expression in pathologic complete response to neoadjuvant chemoradiotherapy for patients with locally advanced rectal adenocarcinoma. Oncotarget. 2016;7(23):35233-40.
103. Min BS, Choi YJ, Pyo HR, Kim H, Seong J, Chung HC, et al. Cyclooxygenase-2 expression in pretreatment biopsy as a predictor of tumor responses after preoperative chemoradiation in rectal cancer. Archives of surgery (Chicago, Ill : 1960). 2008;143(11):1091-7; discussion 7.
104. Kim HJ, Bae SB, Jeong D, Kim ES, Kim CN, Park DG, et al. Upregulation of stromal cell-derived factor 1alpha expression is associated with the resistance to neoadjuvant chemoradiotherapy of locally advanced rectal cancer: angiogenic markers of neoadjuvant chemoradiation. Oncology reports. 2014;32(6):2493-500.
105. Lee-Kong SA, Ruby JA, Chessin DB, Pucciarelli S, Shia J, Riedel ER, et al. Hypoxia-related proteins in patients with rectal cancer undergoing neoadjuvant combined modality therapy. Diseases of the colon and rectum. 2012;55(9):990-5.
106. Shim BY, Jung JH, Lee KM, Kim HJ, Hong SH, Kim SH, et al. Glucose transporter 1 (GLUT1) of anaerobic glycolysis as predictive and prognostic values in neoadjuvant chemoradiotherapy and laparoscopic surgery for locally advanced rectal cancer. International journal of colorectal disease. 2013;28(3):375-83.
107. Lin S, Lai H, Qin Y, Chen J, Lin Y. Thymidine phosphorylase and hypoxia-inducible factor 1-alpha expression in clinical stage II/III rectal cancer: association with response to neoadjuvant chemoradiation therapy and prognosis. International journal of clinical and experimental pathology. 2015;8(9):10680-8.
108. Meng X, Wang R, Huang Z, Zhang J, Feng R, Xu X, et al. Human epidermal growth factor receptor-2 expression in locally advanced rectal cancer: association with response to neoadjuvant therapy and prognosis. Cancer science. 2014;105(7):818-24.
109. McDowell DT, Smith FM, Reynolds JV, Maher SG, Adida C, Crotty P, et al. Increased spontaneous apoptosis, but not survivin expression, is associated with histomorphologic response to neoadjuvant chemoradiation in rectal cancer. International journal of colorectal disease. 2009;24(11):1261-9.
110. Takasu C, Shimada M, Kurita N, Iwata T, Sato H, Nishioka M, et al. Survivin expression can predict the effect of chemoradiotherapy for advanced lower rectal cancer. Int J Clin Oncol. 2013;18(5):869-76.
111. Arber N. Cyclooxygenase-2 Inhibitors in Colorectal Cancer Prevention: Point. 2008;17(8):1852-7.
112. Bengala C, Bettelli S, Bertolini F, Sartori G, Fontana A, Malavasi N, et al. Prognostic role of EGFR gene copy number and KRAS mutation in patients with locally advanced rectal cancer treated with preoperative chemoradiotherapy. British journal of cancer. 2010;103(7):1019-24.
113. Garcia-Aguilar J, Chen Z, Smith DD, Li W, Madoff RD, Cataldo P, et al. Identification of a biomarker profile associated with resistance to neoadjuvant chemoradiation therapy in rectal cancer. Annals of surgery. 2011;254(3):486-92; discussion 92-3.
114. Chen Z, Duldulao MP, Li W, Lee W, Kim J, Garcia-Aguilar J. Molecular diagnosis of response to neoadjuvant chemoradiation therapy in patients with locally advanced rectal cancer. Journal of the American College of Surgeons. 2011;212(6):1008-17.e1.
115. Toiyama Y, Inoue Y, Saigusa S, Okugawa Y, Yokoe T, Tanaka K, et al. Gene expression profiles of epidermal growth factor receptor, vascular endothelial growth factor and hypoxia-inducible factor-1 with special reference to local responsiveness to neoadjuvant chemoradiotherapy and disease recurrence after rectal cancer surgery. Clinical oncology (Royal College of Radiologists (Great Britain)). 2010;22(4):272-80.
116. Lamas MJ, Duran G, Gomez A, Balboa E, Anido U, Bernardez B, et al. X-ray cross-complementing group 1 and thymidylate synthase polymorphisms might predict response to

chemoradiotherapy in rectal cancer patients. International journal of radiation oncology, biology, physics. 2012;82(1):138-44.
117. Lu H, Chu Q, Xie G, Han H, Chen Z, Xu B, et al. Circadian gene expression predicts patient response to neoadjuvant chemoradiation therapy for rectal cancer. International journal of clinical and experimental pathology. 2015;8(9):10985-94.
118. Hur H, Kang J, Kim NK, Min BS, Lee KY, Shin SJ, et al. Thymidylate synthase gene polymorphism affects the response to preoperative 5-fluorouracil chemoradiation therapy in patients with rectal cancer. Int J Radiat Oncol Biol Phys. 2011;81(3):669-76.
119. Nelson B, Carter JV, Eichenberger MR, Netz U, Galandiuk S. Genetic polymorphisms in 5-Fluorouracil-related enzymes predict pathologic response after neoadjuvant chemoradiation for rectal cancer. Surgery. 2016;160(5):1326-32.
120. Paez D, Pare L, Altes A, Sancho-Poch FJ, Petriz L, Garriga J, et al. Thymidylate synthase germline polymorphisms in rectal cancer patients treated with neoadjuvant chemoradiotherapy based on 5-fluorouracil. Journal of cancer research and clinical oncology. 2010;136(11):1681-9.
121. Paez D, Salazar J, Pare L, Pertriz L, Targarona E, del Rio E, et al. Pharmacogenetic study in rectal cancer patients treated with preoperative chemoradiotherapy: polymorphisms in thymidylate synthase, epidermal growth factor receptor, GSTP1, and DNA repair genes. International journal of radiation oncology, biology, physics. 2011;81(5):1319-27.
122. Tan BR, Thomas F, Myerson RJ, Zehnbauer B, Trinkaus K, Malyapa RS, et al. Thymidylate synthase genotype-directed neoadjuvant chemoradiation for patients with rectal adenocarcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(7):875-83.
123. Dreussi E, Cecchin E, Polesel J, Canzonieri V, Agostini M, Boso C, et al. Pharmacogenetics Biomarkers and Their Specific Role in Neoadjuvant Chemoradiotherapy Treatments: An Exploratory Study on Rectal Cancer Patients. International journal of molecular sciences. 2016;17(9).
124. Ho-Pun-Cheung A, Assenat E, Bascoul-Mollevi C, Bibeau F, Boissiere-Michot F, Thezenas S, et al. A large-scale candidate gene approach identifies SNPs in SOD2 and IL13 as predictive markers of response to preoperative chemoradiation in rectal cancer. The pharmacogenomics journal. 2011;11(6):437-43.
125. Xiao L, Yu X, Zhang R, Chang H, Xi S, Xiao W, et al. Can an IL13 -1112 C/T (rs1800925) polymorphism predict responsiveness to neoadjuvant chemoradiotherapy and survival of Chinese Han patients with locally advanced rectal cancer? Oncotarget. 2016;7(23):34149-57.
126. Kim JC, Ha YJ, Roh SA, Cho DH, Choi EY, Kim TW, et al. Novel single-nucleotide polymorphism markers predictive of pathologic response to preoperative chemoradiation therapy in rectal cancer patients. Int J Radiat Oncol Biol Phys. 2013;86(2):350-7.
127. Sebio A, Salazar J, Paez D, Berenguer-Llergo A, Del Rio E, Tobena M, et al. EGFR ligands and DNA repair genes: genomic predictors of complete response after capecitabine-based chemoradiotherapy in locally advanced rectal cancer. The pharmacogenomics journal. 2015;15(1):77-83.
128. Bear HD, Wan W, Robidoux A, Rubin P, Limentani S, White RL, Jr., et al. Using the 21-gene assay from core needle biopsies to choose neoadjuvant therapy for breast cancer: A multicenter trial. Journal of surgical oncology. 2017;115(8):917-23.
129. Brettingham-Moore KH, Duong CP, Greenawalt DM, Heriot AG, Ellul J, Dow CA, et al. Pretreatment transcriptional profiling for predicting response to neoadjuvant chemoradiotherapy in rectal adenocarcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(9):3039-47.
130. Rimkus C, Friederichs J, Boulesteix AL, Theisen J, Mages J, Becker K, et al. Microarray-based prediction of tumor response to neoadjuvant radiochemotherapy of patients with locally advanced rectal cancer. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2008;6(1):53-61.
131. Gantt GA, Chen Y, Dejulius K, Mace AG, Barnholtz-Sloan J, Kalady MF. Gene expression profile is associated with chemoradiation resistance in rectal cancer. Colorectal Dis. 2014;16(1):57-66.

132. Huh JW, Lee JH, Kim HR. Pretreatment expression of 13 molecular markers as a predictor of tumor responses after neoadjuvant chemoradiation in rectal cancer. Annals of surgery. 2014;259(3):508-15.
133. Hur H, Tulina I, Cho MS, Min BS, Koom WS, Lim JS, et al. Biomarker-Based Scoring System for Prediction of Tumor Response After Preoperative Chemoradiotherapy in Rectal Cancer by Reverse Transcriptase Polymerase Chain Reaction Analysis. Dis Colon Rectum. 2016;59(12):1174-82.
134. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.
135. Carames C, Cristobal I, Moreno V, del Puerto L, Moreno I, Rodriguez M, et al. MicroRNA-21 predicts response to preoperative chemoradiotherapy in locally advanced rectal cancer. Int J Colorectal Dis. 2015;30(7):899-906.
136. Carames C, Cristobal I, Moreno V, Marin JP, Gonzalez-Alonso P, Torrejon B, et al. MicroRNA-31 Emerges as a Predictive Biomarker of Pathological Response and Outcome in Locally Advanced Rectal Cancer. International journal of molecular sciences. 2016;17(6).
137. D'Angelo E, Fassan M, Maretto I, Pucciarelli S, Zanon C, Digito M, et al. Serum miR-125b is a non-invasive predictive biomarker of the pre-operative chemoradiotherapy responsiveness in patients with rectal adenocarcinoma. Oncotarget. 2016;7(19):28647-57.
138. D'Angelo E, Zanon C, Sensi F, Digito M, Rugge M, Fassan M, et al. miR-194 as predictive biomarker of responsiveness to neoadjuvant chemoradiotherapy in patients with locally advanced rectal adenocarcinoma. Journal of clinical pathology. 2018;71(4):344-50.
139. Drebber U, Lay M, Wedemeyer I, Vallbohmer D, Bollschweiler E, Brabender J, et al. Altered levels of the onco-microRNA 21 and the tumor-supressor microRNAs 143 and 145 in advanced rectal cancer indicate successful neoadjuvant chemoradiotherapy. International journal of oncology. 2011;39(2):409-15.
140. Della Vittoria Scarpati G, Falcetta F, Carlomagno C, Ubezio P, Marchini S, De Stefano A, et al. A specific miRNA signature correlates with complete pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer. International journal of radiation oncology, biology, physics. 2012;83(4):1113-9.
141. Yu J, Li N, Wang X, Ren H, Wang W, Wang S, et al. Circulating serum microRNA-345 correlates with unfavorable pathological response to preoperative chemoradiotherapy in locally advanced rectal cancer. Oncotarget. 2016;7(39):64233-43.
142. Ha YJ, Kim CW, Roh SA, Cho DH, Park JL, Kim SY, et al. Epigenetic regulation of KLHL34 predictive of pathologic response to preoperative chemoradiation therapy in rectal cancer patients. Int J Radiat Oncol Biol Phys. 2015;91(3):650-8.
143. Molinari C, Casadio V, Foca F, Zingaretti C, Giannini M, Avanzolini A, et al. Gene methylation in rectal cancer: predictive marker of response to chemoradiotherapy? Journal of cellular physiology. 2013;228(12):2343-9.
144. Tsang JS, Vencken S, Sharaf O, Leen E, Kay EW, McNamara DA, et al. Global DNA methylation is altered by neoadjuvant chemoradiotherapy in rectal cancer and may predict response to treatment - A pilot study. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2014;40(11):1459-66.
145. Gonzalez-Gonzalez M, Garcia J, Alcazar JA, Gutierrez ML, Gonzalez LM, Bengoechea O, et al. Association between the cytogenetic profile of tumor cells and response to preoperative radiochemotherapy in locally advanced rectal cancer. Medicine. 2014;93(26):e153.
146. Zaki BI, Suriawinata AA, Eastman AR, Garner KM, Bakhoum SF. Chromosomal instability portends superior response of rectal adenocarcinoma to chemoradiation therapy. Cancer. 2014;120(11):1733-42.
147. Molinari C, Ballardini M, Teodorani N, Giannini M, Zoli W, Emiliani E, et al. Genomic alterations in rectal tumors and response to neoadjuvant chemoradiotherapy: an exploratory study. Radiat. 2011;6:161.
148. Chee CG, Kim YH, Lee KH, Lee YJ, Park JH, Lee HS, et al. CT texture analysis in patients with locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy: A potential imaging biomarker for treatment response and prognosis. PloS one. 2017;12(8):e0182883.

149. Chen YG, Chen MQ, Guo YY, Li SC, Wu JX, Xu BH. Apparent Diffusion Coefficient Predicts Pathology Complete Response of Rectal Cancer Treated with Neoadjuvant Chemoradiotherapy. PloS one. 2016;11(4):e0153944.
150. Kim SH, Lee JY, Lee JM, Han JK, Choi BI. Apparent diffusion coefficient for evaluating tumour response to neoadjuvant chemoradiation therapy for locally advanced rectal cancer. European radiology. 2011;21(5):987-95.
151. Sathyakumar K, Chandramohan A, Masih D, Jesudasan MR, Pulimood A, Eapen A. Best MRI predictors of complete response to neoadjuvant chemoradiation in locally advanced rectal cancer. The British journal of radiology. 2016;89(1060):20150328.
152. Iannicelli E, Di Pietropaolo M, Pilozzi E, Osti MF, Valentino M, Masoni L, et al. Value of diffusion-weighted MRI and apparent diffusion coefficient measurements for predicting the response of locally advanced rectal cancer to neoadjuvant chemoradiotherapy. Abdominal radiology (New York). 2016;41(10):1906-17.
153. Intven M, Reerink O, Philippens ME. Diffusion-weighted MRI in locally advanced rectal cancer : pathological response prediction after neo-adjuvant radiochemotherapy. Strahlenther Onkol. 2013;189(2):117-22.
154. Choi MH, Oh SN, Rha SE, Choi JI, Lee SH, Jang HS, et al. Diffusion-weighted imaging: Apparent diffusion coefficient histogram analysis for detecting pathologic complete response to chemoradiotherapy in locally advanced rectal cancer. Journal of magnetic resonance imaging : JMRI. 2016;44(1):212-20.
155. Zhu HB, Zhang XY, Zhou XH, Li XT, Liu YL, Wang S, et al. Assessment of pathological complete response to preoperative chemoradiotherapy by means of multiple mathematical models of diffusion-weighted MRI in locally advanced rectal cancer: A prospective single-center study. Journal of magnetic resonance imaging : JMRI. 2017;46(1):175-83.
156. Tong T, Sun Y, Gollub MJ, Peng W, Cai S, Zhang Z, et al. Dynamic contrast-enhanced MRI: Use in predicting pathological complete response to neoadjuvant chemoradiation in locally advanced rectal cancer. Journal of magnetic resonance imaging : JMRI. 2015;42(3):673-80.
157. Martens MH, Subhani S, Heijnen LA, Lambregts DM, Buijsen J, Maas M, et al. Can perfusion MRI predict response to preoperative treatment in rectal cancer? Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology. 2015;114(2):218-23.
158. DeVries AF, Piringer G, Kremser C, Judmaier W, Saely CH, Lukas P, et al. Pretreatment evaluation of microcirculation by dynamic contrast-enhanced magnetic resonance imaging predicts survival in primary rectal cancer patients. International journal of radiation oncology, biology, physics. 2014;90(5):1161-7.
159. Cusumano D, Dinapoli N, Boldrini L, Chiloiro G, Gatta R, Masciocchi C, et al. Fractal-based radiomic approach to predict complete pathological response after chemo-radiotherapy in rectal cancer. Radiol Med (Torino). 2018;123(4):286-95.
160. Palmisano A, Esposito A, Di Chiara A, Ambrosi A, Passoni P, Slim N, et al. Could early tumour volume changes assessed on morphological MRI predict the response to chemoradiation therapy in locally-advanced rectal cancer? Clinical Radiology. 2018;73(6):555-63.
161. Dinapoli N, Barbaro B, Gatta R, Chiloiro G, Casa C, Masciocchi C, et al. Magnetic Resonance, Vendor-independent, Intensity Histogram Analysis Predicting Pathologic Complete Response After Radiochemotherapy of Rectal Cancer. International Journal of Radiation Oncology Biology Physics. 2018.
162. Tong T, Sun Y, Cai S, Zhang Z, Gu Y. Extramural depth of rectal cancer tumor invasion at thin-section MRI: predicting treatment response to neoadjuvant chemoradiation. Oncotarget. 2015;6(30):30277-86.
163. Wilkins S, Haydon A, Porter I, Oliva K, Staples M, Carne P, et al. Complete Pathological Response After Neoadjuvant Long-Course Chemoradiotherapy for Rectal Cancer and Its Relationship to the Degree of T3 Mesorectal Invasion. Diseases of the colon and rectum. 2016;59(5):361-8.
164. Liberti MV, Locasale JW. The Warburg Effect: How Does it Benefit Cancer Cells? Trends Biochem Sci. 2016;41(3):211-8.

165. Withofs N, Martinive P, Vanderick J, Bletard N, Scagnol I, Mievis F, et al. [(18)F]FPRGD2 PET/CT imaging of integrin alphavbeta3 levels in patients with locally advanced rectal carcinoma. Eur J Nucl Med Mol Imaging. 2016;43(4):654-62.
166. Chennupati SK, Quon A, Kamaya A, Pai RK, La T, Krakow TE, et al. Positron emission tomography for predicting pathologic response after neoadjuvant chemoradiotherapy for locally advanced rectal cancer. American journal of clinical oncology. 2012;35(4):334-9.
167. Guerra L, Niespolo R, Di Pisa G, Ippolito D, De Ponti E, Terrevazzi S, et al. Change in glucose metabolism measured by 18F-FDG PET/CT as a predictor of histopathologic response to neoadjuvant treatment in rectal cancer. Abdom Imaging. 2011;36(1):38-45.
168. Koo PJ, Kim SJ, Chang S, Kwak JJ. Interim Fluorine-18 Fluorodeoxyglucose Positron Emission Tomography/Computed Tomography to Predict Pathologic Response to Preoperative Chemoradiotherapy and Prognosis in Patients With Locally Advanced Rectal Cancer. Clin Colorectal Cancer. 2016;15(4):e213-e9.
169. Lee SJ, Kim JG, Lee SW, Chae YS, Kang BW, Lee YJ, et al. Clinical implications of initial FDG-PET/CT in locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy. Cancer chemotherapy and pharmacology. 2013;71(5):1201-7.
170. Maffione AM, Ferretti A, Chondrogiannis S, Rampin L, Marzola MC, Grassetto G, et al. Proposal of a new 18F-FDG PET/CT predictor of response in rectal cancer treated by neoadjuvant chemoradiation therapy and comparison with PERCIST criteria. Clinical nuclear medicine. 2013;38(10):795-7.
171. Martoni AA, Di Fabio F, Pinto C, Castellucci P, Pini S, Ceccarelli C, et al. Prospective study on the FDG-PET/CT predictive and prognostic values in patients treated with neoadjuvant chemoradiation therapy and radical surgery for locally advanced rectal cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2011;22(3):650-6.
172. Perez RO, Habr-Gama A, Sao Juliao GP, Lynn PB, Sabbagh C, Proscurshim I, et al. Predicting complete response to neoadjuvant CRT for distal rectal cancer using sequential PET/CT imaging. Techniques in coloproctology. 2014;18(8):699-708.
173. Glimelius B, Gronberg H, Jarhult J, Wallgren A, Cavallin-Stahl E. A systematic overview of radiation therapy effects in rectal cancer. Acta oncologica (Stockholm, Sweden). 2003;42(5-6):476-92.
174. Choi E, Kim JH, Kim OB, Kim MY, Oh YK, Baek SG. Predictors of pathologic complete response after preoperative concurrent chemoradiotherapy of rectal cancer: a single center experience. Radiation oncology journal. 2016;34(2):106-12.
175. Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, et al. Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. Thorax. 2003;58(5):377-82.
176. Jones AE, Trzeciak S, Kline JA. The Sequential Organ Failure Assessment score for predicting outcome in patients with severe sepsis and evidence of hypoperfusion at the time of emergency department presentation. Critical care medicine. 2009;37(5):1649-54.
177. Cho JH, Kim TN, Chung HH, Kim KH. Comparison of scoring systems in predicting the severity of acute pancreatitis. World J Gastroenterol. 2015;21(8):2387-94.
178. Myerson RJ, Singh A, Birnbaum EH, Fry RD, Fleshman JW, Kodner IJ, et al. Pretreatment clinical findings predict outcome for patients receiving preoperative radiation for rectal cancer. International journal of radiation oncology, biology, physics. 2001;50(3):665-74.
179. Edge S, Byrd, DR, Compton, CC, Fritz, AG, Greene, FL, Trotti, A, editors. AJCC cancer staging manual (7th ed). New York, New York.: Springer; 2010.
180. Kenney BC, Jain D. Identification of lymphatics within the colonic lamina propria in inflammation and neoplasia using the monoclonal antibody D2-40. The Yale journal of biology and medicine. 2008;81(3):103-13.
181. Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012;327(1-2):48-60.
182. Kam WW, Banati RB. Effects of ionizing radiation on mitochondria. Free Radic Biol Med. 2013;65:607-19.

183. Rockwell S, Dobrucki IT, Kim EY, Marrison ST, Vu VT. Hypoxia and radiation therapy: past history, ongoing research, and future promise. Curr Mol Med. 2009;9(4):442-58.
184. Hirose K, Longo DL, Oppenheim JJ, Matsushima K. Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. FASEB J. 1993;7(2):361-8.
185. Motoori S, Majima HJ, Ebara M, Kato H, Hirai F, Kakinuma S, et al. Overexpression of mitochondrial manganese superoxide dismutase protects against radiation-induced cell death in the human hepatocellular carcinoma cell line HLE. Cancer Res. 2001;61(14):5382-8.
186. Epperly MW, Melendez JA, Zhang X, Nie S, Pearce L, Peterson J, et al. Mitochondrial targeting of a catalase transgene product by plasmid liposomes increases radioresistance in vitro and in vivo. Radiat Res. 2009;171(5):588-95.
187. Petkau A. Role of superoxide dismutase in modification of radiation injury. Br J Cancer Suppl. 1987;8:87-95.
188. Mitchell JB, Biaglow JE, Russo A. Role of glutathione and other endogenous thiols in radiation protection. Pharmacology & Therapeutics. 1988;39(1):269-74.
189. Rhee SG, Kil IS. Multiple Functions and Regulation of Mammalian Peroxiredoxins. Annu Rev Biochem. 2017;86:749-75.
190. Perkins A, Nelson KJ, Parsonage D, Poole LB, Karplus PA. Peroxiredoxins: guardians against oxidative stress and modulators of peroxide signaling. Trends Biochem Sci. 2015;40(8):435-45.
191. Hampton MB, Vick KA, Skoko JJ, Neumann CA. Peroxiredoxin Involvement in the Initiation and Progression of Human Cancer. Antioxid Redox Signal. 2018;28(7):591-608.
192. Brown KK, Cox AG, Hampton MB. Mitochondrial respiratory chain involvement in peroxiredoxin 3 oxidation by phenethyl isothiocyanate and auranofin. FEBS Letters. 2010;584(6):1257-62.
193. Poynton RA, Hampton MB. Peroxiredoxins as biomarkers of oxidative stress. Biochim Biophys Acta. 2014;1840(2):906-12.
194. National Cancer Institute. Cancer Stat Facts: Colorectal Cancer 2015 [Available from: https://seer.cancer.gov/statfacts/html/colorect.html.
195. Petersen SH, Harling H, Kirkeby LT, Wille-Jorgensen P, Mocellin S. Postoperative adjuvant chemotherapy in rectal cancer operated for cure. Cochrane Database Syst Rev. 2012(3):Cd004078.
196. Goldfeder A. The integrity of cytoplasmic ultrastructures: a factor in cellular radiosensitivity. Cellular Radiation Biology, Symposium on Fundamental Cancer Research (MD Anderson Hospital and Tumor Institute: Baltimore: Williams and Wilkins). 1965:539-46.
197. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981;290(5806):457-65.
198. Chomyn A, Mariottini P, Cleeter MW, Ragan CI, Matsuno-Yagi A, Hatefi Y, et al. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. Nature. 1985;314(6012):592-7.
199. Chomyn A, Cleeter MW, Ragan CI, Riley M, Doolittle RF, Attardi G. URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. Science. 1986;234(4776):614-8.
200. Macreadie IG, Novitski CE, Maxwell RJ, John U, Ooi BG, McMullen GL, et al. Biogenesis of mitochondria: the mitochondrial gene (aap1) coding for mitochondrial ATPase subunit 8 in Saccharomyces cerevisiae. Nucleic Acids Res. 1983;11(13):4435-51.
201. Murphy Michael P. How mitochondria produce reactive oxygen species. Biochemical Journal. 2009;417(Pt 1):1-13.
202. Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. Cancer Res. 2001;61(10):3894-901.
203. Richardson RB, Harper ME. Mitochondrial stress controls the radiosensitivity of the oxygen effect: Implications for radiotherapy. Oncotarget. 2016;7(16):21469-83.
204. De Bont R, van Larebeke N. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis. 2004;19(3):169-85.

205. Sun J, Chen Y, Li M, Ge Z. Role of antioxidant enzymes on ionizing radiation resistance. Free Radic Biol Med. 1998;24(4):586-93.
206. Oberley LW, St Clair DK, Autor AP, Oberley TD. Increase in manganese superoxide dismutase activity in the mouse heart after X-irradiation. Arch Biochem Biophys. 1987;254(1):69-80.
207. Akashi M, Hachiya M, Paquette RL, Osawa Y, Shimizu S, Suzuki G. Irradiation increases manganese superoxide dismutase mRNA levels in human fibroblasts. Possible mechanisms for its accumulation. J Biol Chem. 1995;270(26):15864-9.
208. Fuchs-Tarlovsky V. Role of antioxidants in cancer therapy. Nutrition. 2013;29(1):15-21.
209. Spencer A, Horvath N, Gibson J, Prince HM, Herrmann R, Bashford J, et al. Prospective randomised trial of amifostine cytoprotection in myeloma patients undergoing high-dose melphalan conditioned autologous stem cell transplantation. Bone Marrow Transplant. 2005;35(10):971-7.
210. Murphy A, Testa K, Berkelhammer J, Hopkins S, Loo G. Impact of antioxidants on the ability of phenolic phytochemicals to kill HCT116 colon cancer cells. Free radical research. 2014;48(3):313-21.
211. Zhang B, Wang Y, Su Y. Peroxiredoxins, a novel target in cancer radiotherapy. Cancer Lett. 2009;286(2):154-60.
212. An JH, Kim J, Seong J. Redox signaling by ionizing radiation in mouse liver. Ann N Y Acad Sci. 2004;1030:86-94.
213. Lee K, Park JS, Kim YJ, Soo Lee YS, Sook Hwang TS, Kim DJ, et al. Differential expression of Prx I and II in mouse testis and their up-regulation by radiation. Biochem Biophys Res Commun. 2002;296(2):337-42.
214. Zhang B, Su Y, Ai G, Wang Y, Wang T, Wang F. Involvement of peroxiredoxin I in protecting cells from radiation-induced death. J Radiat Res. 2005;46(3):305-12.
215. Chen WC, McBride WH, Iwamoto KS, Barber CL, Wang CC, Oh YT, et al. Induction of radioprotective peroxiredoxin-I by ionizing irradiation. J Neurosci Res. 2002;70(6):794-8.
216. Park SH, Chung YM, Lee YS, Kim HJ, Kim JS, Chae HZ, et al. Antisense of human peroxiredoxin II enhances radiation-induced cell death. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(12):4915-20.
217. Cerda MB, Lloyd R, Batalla M, Giannoni F, Casal M, Policastro L. Silencing peroxiredoxin-2 sensitizes human colorectal cancer cells to ionizing radiation and oxaliplatin. Cancer Lett. 2017;388:312-9.
218. Zhang B, Wang Y, Liu K, Yang X, Song M, Wang Y, et al. Adenovirus-mediated transfer of siRNA against peroxiredoxin I enhances the radiosensitivity of human intestinal cancer. Biochem Pharmacol. 2008;75(3):660-7.
219. Wu XY, Fu ZX, Wang XH. Peroxiredoxins in colorectal neoplasms. Histol Histopathol. 2010;25(10):1297-303.
220. Chen MF, Lee KD, Yeh CH, Chen WC, Huang WS, Chin CC, et al. Role of peroxiredoxin I in rectal cancer and related to p53 status. International journal of radiation oncology, biology, physics. 2010;78(3):868-78.
221. Peng L, Wang R, Shang J, Xiong Y, Fu Z. Peroxiredoxin 2 is associated with colorectal cancer progression and poor survival of patients. Oncotarget. 2017;8(9):15057-70.
222. Lu W, Fu Z, Wang H, Feng J, Wei J, Guo J. Peroxiredoxin 2 is upregulated in colorectal cancer and contributes to colorectal cancer cells' survival by protecting cells from oxidative stress. Molecular and cellular biochemistry. 2014;387(1-2):261-70.
223. Kang DH, Lee DJ, Lee S, Lee SY, Jun Y, Kim Y, et al. Interaction of tankyrase and peroxiredoxin II is indispensable for the survival of colorectal cancer cells. Nature communications. 2017;8(1):40.
224. Lu W, Fu Z, Wang H, Feng J, Wei J, Guo J. Peroxiredoxin 2 knockdown by RNA interference inhibits the growth of colorectal cancer cells by downregulating Wnt/beta-catenin signaling. Cancer Lett. 2014;343(2):190-9.
225. Ji D, Li M, Zhan T, Yao Y, Shen J, Tian H, et al. Prognostic role of serum AZGP1, PEDF and PRDX2 in colorectal cancer patients. Carcinogenesis. 2013;34(6):1265-72.
226. Xu J, Zhang S, Wang R, Wu X, Zeng L, Fu Z. Knockdown of PRDX2 sensitizes colon cancer cells to 5-FU by suppressing the PI3K/AKT signaling pathway. Bioscience reports. 2017;37(3).

227. Song IS, Jeong YJ, Jeong SH, Heo HJ, Kim HK, Bae KB, et al. FOXM1-Induced PRX3 Regulates Stemness and Survival of Colon Cancer Cells via Maintenance of Mitochondrial Function. Gastroenterology. 2015;149(4):1006-16 e9.
228. Yi N, Xiao MB, Ni WK, Jiang F, Lu CH, Ni RZ. High expression of peroxiredoxin 4 affects the survival time of colorectal cancer patients, but is not an independent unfavorable prognostic factor. Molecular and clinical oncology. 2014;2(5):767-72.
229. Repetto O, De Re V, De Paoli A, Belluco C, Alessandrini L, Canzonieri V, et al. Identification of protein clusters predictive of tumor response in rectal cancer patients receiving neoadjuvant chemo-radiotherapy. Oncotarget. 2017;8(17):28328-41.
230. Ahn HM, Yoo JW, Lee S, Lee HJ, Lee HS, Lee DS. Peroxiredoxin 5 promotes the epithelial-mesenchymal transition in colon cancer. Biochem Biophys Res Commun. 2017;487(3):580-6.
231. Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. Biochem J. 2009;425(2):313-25.
232. Huang BK, Langford TF, Sikes HD. Using Sensors and Generators of H(2)O(2) to Elucidate the Toxicity Mechanism of Piperlongumine and Phenethyl Isothiocyanate. Antioxidants & Redox Signaling. 2016;24(16):924-38.
233. Haraldsen JD, Liu G, Botting CH, Walton JG, Storm J, Phalen TJ, et al. IDENTIFICATION OF CONOIDIN A AS A COVALENT INHIBITOR OF PEROXIREDOXIN II. Organic & biomolecular chemistry. 2009;7:3040-8.
234. Liu G, Botting Catherine H, Evans Kathryn M, Walton Jeffrey AG, Xu G, Slawin Alexandra MZ, et al. Optimisation of Conoidin A, a Peroxiredoxin Inhibitor. ChemMedChem. 2009;5(1):41-5.
235. Liu CX, Yin QQ, Zhou HC, Wu YL, Pu JX, Xia L, et al. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. Nat Chem Biol. 2012;8(5):486-93.
236. Newick K, Cunniff B, Preston K, Held P, Arbiser J, Pass H, et al. Peroxiredoxin 3 is a redox-dependent target of thiostrepton in malignant mesothelioma cells. PloS one. 2012;7(6):e39404.
237. Lopert P, Day BJ, Patel M. Thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells. PloS one. 2012;7(11):e50683.
238. Stafford WC, Peng X, Olofsson MH, Zhang X, Luci DK, Lu L, et al. Irreversible inhibition of cytosolic thioredoxin reductase 1 as a mechanistic basis for anticancer therapy. Science translational medicine. 2018;10(428).
239. Wang F, Lin F, Zhang P, Ni W, Bi L, Wu J, et al. Thioredoxin-1 inhibitor, 1-methylpropyl 2-imidazolyl disulfide, inhibits the growth, migration and invasion of colorectal cancer cell lines. Oncology reports. 2015;33(2):967-73.
240. Karplus PA. A primer on peroxiredoxin biochemistry. Free Radic Biol Med. 2015;80:183-90.
241. Bayer SB, Maghzal G, Stocker R, Hampton MB, Winterbourn CC. Neutrophil-mediated oxidation of erythrocyte peroxiredoxin 2 as a potential marker of oxidative stress in inflammation. Faseb j. 2013;27(8):3315-22.
242. Kumar V, Kitaeff N, Hampton MB, Cannell MB, Winterbourn CC. Reversible oxidation of mitochondrial peroxiredoxin 3 in mouse heart subjected to ischemia and reperfusion. FEBS Letters. 2009;583(6):997-1000.
243. Weber D, Davies MJ, Grune T. Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. Redox Biol. 2015;5:367-80.
244. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chimica Acta. 2003;329(1):23-38.
245. Winterbourn CC, Bonham MJ, Buss H, Abu-Zidan FM, Windsor JA. Elevated protein carbonyls as plasma markers of oxidative stress in acute pancreatitis. Pancreatology. 2003;3(5):375-82.
246. Wang Z, Li S, Cao Y, Tian X, Zeng R, Liao DF, et al. Oxidative Stress and Carbonyl Lesions in Ulcerative Colitis and Associated Colorectal Cancer. Oxidative medicine and cellular longevity. 2016;2016:9875298.
247. Fernando N, Wickremesinghe S, Niloofa R, Rodrigo C, Karunanayake L, de Silva HJ, et al. Protein Carbonyl as a Biomarker of Oxidative Stress in Severe Leptospirosis, and Its Usefulness in Differentiating Leptospirosis from Dengue Infections. PloS one. 2016;11(6):e0156085.

248. Biocell Corporation. BioCell Protein Carbonyl Assay Kit Instruction Manual Auckland, New Zealand2018 [Available from: https://biocellcorp.co.nz/wp-content/uploads/2018/10/kit-instructions-batch-p131-1.pdf.
249. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 1991;51(3):794-8.
250. Danesh BJ, Burke M, Newman J, Aylott A, Whitfield P, Cotton PB. Comparison of weight, depth, and diagnostic adequacy of specimens obtained with 16 different biopsy forceps designed for upper gastrointestinal endoscopy. Gut. 1985;26(3):227-31.
251. Carini F, Mazzola M, Rappa F, Jurjus A, Geagea AG, Al Kattar S, et al. Colorectal Carcinogenesis: Role of Oxidative Stress and Antioxidants. Anticancer Res. 2017;37(9):4759-66.
252. Bennett MH, Feldmeier J, Smee R, Milross C. Hyperbaric oxygenation for tumour sensitisation to radiotherapy. Cochrane Database of Systematic Reviews. 2018(4).
253. Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015;21(11):1350-6.
254. Kawakami H, Zaanan A, Sinicrope FA. MSI testing and its role in the management of colorectal cancer. Current treatment options in oncology. 2015;16(7):30-.
255. Saridaki Z, Souglakos J, Georgoulias V. Prognostic and predictive significance of MSI in stages II/III colon cancer. World Journal of Gastroenterology : WJG. 2014;20(22):6809-14.
256. Chen J, Ye Y, Sun H, Shi G. Association between KRAS codon 13 mutations and clinical response to anti-EGFR treatment in patients with metastatic colorectal cancer: results from a meta-analysis. Cancer chemotherapy and pharmacology. 2013;71(1):265-72.
257. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. New England Journal of Medicine. 2015;372(26):2509-20.
258. Agyeman AA, Ofori-Asenso R. Perspective: Does personalized medicine hold the future for medicine? Journal of pharmacy & bioallied sciences. 2015;7(3):239-44.
259. Lee YY, Li CF, Lin CY, Lee SW, Sheu MJ, Lin LC, et al. Overexpression of CPS1 is an independent negative prognosticator in rectal cancers receiving concurrent chemoradiotherapy. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35(11):11097-105.
260. Ahmed MA, Selzer E, Dorr W, Jomrich G, Harpain F, Silberhumer GR, et al. Fibroblast growth factor receptor 4 induced resistance to radiation therapy in colorectal cancer. Oncotarget. 2016;7(43):69976-90.
261. Cebrian A, Gomez Del Pulgar T, Fernandez-Acenero MJ, Borrero-Palacios A, Del Puerto-Nevado L, Martinez-Useros J, et al. Decreased PLK1 expression denotes therapy resistance and unfavourable disease-free survival in rectal cancer patients receiving neoadjuvant chemoradiotherapy. Pathology, research and practice. 2016;212(12):1133-7.
262. Lin CY, Sheu MJ, Li CF, Lee SW, Lin LC, Wang YF, et al. Deficiency in asparagine synthetase expression in rectal cancers receiving concurrent chemoradiotherapy: negative prognostic impact and therapeutic relevance. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35(7):6823-30.
263. Lin CY, Tian YF, Wu LC, Chen LT, Lin LC, Hsing CH, et al. Rsf-1 expression in rectal cancer: with special emphasis on the independent prognostic value after neoadjuvant chemoradiation. Journal of clinical pathology. 2012;65(8):687-92.
264. Sheu MJ, Li CF, Lin CY, Lee SW, Lin LC, Chen TJ, et al. Overexpression of ANXA1 confers independent negative prognostic impact in rectal cancers receiving concurrent chemoradiotherapy. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35(8):7755-63.
265. Farkas R, Pozsgai E, Schally AV, Szigeti A, Szigeti E, Laszlo Z, et al. Possible predictors of histopathological response to neoadjuvant chemoradiotherapy for rectal cancer. Journal of cancer research and clinical oncology. 2012;138(3):387-95.
266. Shim BY, Sun S, Won HS, Lee MA, Hong SU, Jung JH, et al. Role of autophagy-related protein expression in patients with rectal cancer treated with neoadjuvant chemoradiotherapy. BMC Cancer. 2016;16:207.

267. Sprenger T, Conradi LC, Beissbarth T, Ermert H, Homayounfar K, Middel P, et al. Enrichment of CD133-expressing cells in rectal cancers treated with preoperative radiochemotherapy is an independent marker for metastasis and survival. Cancer. 2013;119(1):26-35.
268. Gomez Del Pulgar T, Cebrian A, Fernandez-Acenero MJ, Borrero-Palacios A, Del Puerto-Nevado L, Martinez-Useros J, et al. Focal adhesion kinase: predictor of tumour response and risk factor for recurrence after neoadjuvant chemoradiation in rectal cancer. Journal of cellular and molecular medicine. 2016;20(9):1729-36.
269. Yan H, Yu J, Wang R, Jiang S, Zhu K, Mu D, et al. Prognostic value of Smac expression in rectal cancer patients treated with neoadjuvant therapy. Medical oncology (Northwood, London, England). 2012;29(1):168-73.
270. Goncalves-Ribeiro S, Sanz-Pamplona R, Vidal A, Sanjuan X, Guillen Diaz-Maroto N, Soriano A, et al. Prediction of pathological response to neoadjuvant treatment in rectal cancer with a two-protein immunohistochemical score derived from stromal gene-profiling. Ann Oncol. 2017;28(9):2160-8.
271. O'Neil BH, Funkhouser WK, Calvo BF, Meyers MO, Kim HJ, Goldberg RM, et al. Nuclear factor kappa-light chain-enhancer of activated B cells is activated by radiotherapy and is prognostic for overall survival in patients with rectal cancer treated with preoperative fluorouracil-based chemoradiotheraphy. Int J Radiat Oncol Biol Phys. 2011;80(3):705-11.
272. Lee HY, Jung JH, Cho HM, Kim SH, Lee KM, Kim HJ, et al. GRP78 Protein Expression as Prognostic Values in Neoadjuvant Chemoradiotherapy and Laparoscopic Surgery for Locally Advanced Rectal Cancer. Cancer research and treatment : official journal of Korean Cancer Association. 2015;47(4):804-12.
273. He HL, Lee YE, Chen HP, Hsing CH, Chang IW, Shiue YL, et al. Overexpression of DNAJC12 predicts poor response to neoadjuvant concurrent chemoradiotherapy in patients with rectal cancer. Experimental and molecular pathology. 2015;98(3):338-45.
274. He HL, Lee YE, Shiue YL, Lee SW, Lin LC, Chen TJ, et al. Overexpression of REG4 confers an independent negative prognosticator in rectal cancers receiving concurrent chemoradiotherapy. Journal of surgical oncology. 2014;110(8):1002-10.
275. Senetta R, Duregon E, Sonetto C, Spadi R, Mistrangelo M, Racca P, et al. YKL-40/c-Met expression in rectal cancer biopsies predicts tumor regression following neoadjuvant chemoradiotherapy: a multi-institutional study. PloS one. 2015;10(4):e0123759.
276. He HL, Lee YE, Shiue YL, Lee SW, Lin LC, Chen TJ, et al. PLA2G2A overexpression is associated with poor therapeutic response and inferior outcome in rectal cancer patients receiving neoadjuvant concurrent chemoradiotherapy. Histopathology. 2015;66(7):991-1002.
277. Hongo K, Kazama S, Tsuno NH, Ishihara S, Sunami E, Kitayama J, et al. Immunohistochemical detection of high-mobility group box 1 correlates with resistance of preoperative chemoradiotherapy for lower rectal cancer: a retrospective study. World J Surg Oncol. 2015;13:7.
278. Lee YE, He HL, Shiue YL, Lee SW, Lin LC, Wu TF, et al. The prognostic impact of lipid biosynthesis-associated markers, HSD17B2 and HMGCS2, in rectal cancer treated with neoadjuvant concurrent chemoradiotherapy. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015;36(10):7675-83.