AN INVESTIGATION INTO THE INFLUENCE OF THE SYSTEMIC INFLAMMATORY RESPONSE ON TREATMENT RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY IN RECTAL CANCER

BY

HARIKRISHNAN S. NAIR

MBChB MRCS FRCS

A THESIS SUBMITTED IN THE FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF MEDICINE (MD)

TO

THE UNIVERSITY OF GLASGOW

From research conducted in the Academic Unit of Surgery, Glasgow Royal Infirmary, College of Medical and Veterinary Life Sciences, University of Glasgow

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABBREVIATION	FULL FORM
AFAP	Attenuated Familial Adenomatous Polyposis
APC	adenomatous polyposis coli
APR	Abdominoperineal resection
AUC	Area under curve
BMI	Body Mass Index
cCR	complete clinical response
CEA	Carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus Molecular Subtypes
CRC	Colorectal cancer
CRP	C-Reactive protein
CRT	chemoradiotherapy
CT	Computed Tomography
CTC	Computed tomographic colonography
CTCAE	Common Terminology Criteria for Adverse Even

DFS	Disease free survival
DNA	Deoxyribonucleic acid
DWI	diffusion weighted imaging
EGFR	Epidermal growth factor receptors
EMVI	Extramural venous invasion
EUS	endoscopic ultrasound
FAP	Familial adenomatous Polyposis
FIT	Faecal immunochemical testing
FOBT	Faecal occult blood tests
FS	Flexible sigmoidoscopy
Hb	Haemoglobin
HNPCC	Hereditary Non Polyposis Colorectal Cancer
IFN-γ)	interferon gamma
IGFBP2	insulin-like growth factor binding protein 2
IMA	Inferior Mesenteric Artery
IWWD	International Watch and Wait Database
KRAS	Kirsten rat sarcoma virus
LARC	Locally advanced rectal cancer

LCRT	long course chemoradiotherapy
MAP	MUTYH associated polyposis
MCP	monocyte chemoattractant protein
MDT	multidisciplinary team
mGPS	modified Glasgow Prognostic Score
MMR	mismatch repair
MRF	mesorectal fascia
MRI	Magnetic Resonance Imaging
mrTRG	MRI tumour regression grade
MSI	Microsatellite instability
NAR	neoadjuvant rectal
NCCN	National Comprehensive Cancer Network
nCRT	neoadjuvant CRT
NLR	neutrophil lymphocyte ratio
NOTCH3	neurogenic locus notch homolog protein 3
NPS	neutrophil-platelet score
OPRA	Organ preservation of Rectal adenocarcinoma
OS	overall survival

pCR	pathological complete response
PDGFBB	platelet derived growth factor BB
PET-CT	Positron emission tomography - computed tomography
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PLR	platelet-to-lymphocyte ratio
RC	Rectal cancer
RFS	recurrence free survival
RT	radiotherapy
SCRT	short course radiotherapy
SII	systemic inflammatory index
SIR	systemic inflammatory repones
SSA	Sessile serrated adenomas
TGF-β	transforming growth factor beta
Th1	Helper T cells 1
TME	Total mesorectal excision
TNF-α	tumour necrosis factor alpha
TNM	Tumour, node, metastasis

Treg	regulatory T cells
TRG	Tumour regression grade
VEGFR2	vascular endothelial growth factor receptor 2
WBC	White blood count

Acknowledgements

I would like to firstly thank my supervisors Professor Campbell Roxburgh and Professor Joanne Edwards for their support, guidance and patience during my research and afterwards.

I would like to the thank the following individuals for their guidance, support and expertise during my research:

Mr Stephen McSorley, Mr Colin Steele, Mr James Park, Dr Janet Graham, Dr Alec McDonald, Dr Jean Quinn, Dr Kathryn Pennell, Professor Donald McMillan and Professor Paul Horgan.

I would like to thank all the study patients and their treating clinicians without whom none of this would be possible.

I would like to thank my family, especially my wife Anjali and daughters Anoushka and Ahaana who have been a tremendous support and exceptionally patient throughout my research period.

Declaration

I declare that the work presented in this thesis was undertaken by myself.

In addition, the following individuals contributed:

Dr Janet Graham and Mr Colin Steele for facilitating access to the main patient database for this study.

Mr Stephen McSorley for access to Glasgow Royal Infirmary dataset used to create the main patient database.

Professor Roxburgh for his role as principal investigator and creation of the PRIME study protocol.

Ethics approval for this study was granted by the Caldicott Guardian and West of Scotland Research Ethics Committee.

Abstract

An abnormal systemic inflammatory response is associated with adverse short and long term outcomes in cancer. Systemic inflammation can be a surrogate maker of the interaction between host immune response and tumour. Systemic inflammation can influence treatment response to chemoradiotherapy. At present there is no reliable biomarker of response to chemoradiotherapy in rectal cancer. This thesis examines the influence of the systemic inflammatory response on treatment response to neoadjuvant chemoradiotherapy in rectal cancer.

A dataset of patients receiving neoadjuvant long course chemoradiotherapy (CRT) for non-metastatic disease followed by potentially curative resection for rectal cancer was compiled from two prospectively databases of patients treated at Glasgow Royal Infirmary from 2008-2014 and the wider West of Scotland between 2014-2016. Blood results and clinic-pathological data for these patients were collected from electronic patient records to create a comprehensive dataset. Biomarkers of systemic inflammation included: differential blood count; neutrophil to lymphocyte ratio (NLR); haemoglobin; C reactive protein; albumin; and modified Glasgow Prognostic Score(mGPS). Treatment response to chemoradiotherapy was quantified with tumour regression grade, pathological complete response and the neoadjuvant rectal score.

I observed white cell count (WCC), NLR and mGPS were not associated with treatment response. Lower haemoglobin and elevated CEA were associated with poorer tumour response. There was no association with changes in WCC, NLR, CRP and tumour response. I observed the development of lymphopenia during treatment but no association with tumour response. I observed baseline anaemia was associated with poorer tumour response and an association between anaemia and systemic inflammation. A significant proportion of my time was in the recruitment and coordination of sample collection for a

novel pilot study for the feasibility of protocolised blood and tumour sampling during neoadjuvant therapy.

I have not demonstrated an association between serum markers of systemic inflammation and treatment response. I have demonstrated anaemia is a marker of poor response and the association between anaemia and systemic inflammation. This highlights the difficulty in measurements of the systemic inflammatory response from routine blood tests and the importance of more detailed study of markers of systemic inflammatory response which are being done in research settings rather than routine clinical practice. This would help identify reliable biomarkers of treatment response to neoadjuvant chemotherapy and organ preservation strategies.

1 Introduction

1.1 Epidemiology of Rectal Cancer

Colorectal cancer is the 3rd commonest cancer and the second commonest cause of cancer related mortality in the UK (CRUK cancer statistics). The number of new colorectal cancers (CRC) diagnosed each year in the UK in the region of 42,800 cases and set rise to 47700 by 2038-2040. The most common site encompassing a third of these cancers are within in the rectum. [1] The incidence of rectal cancer is highest in Japan and Eastern Europe with lower rates in Africa and Asia. The incidence has been stabilising or falling in higher risk regions but increasing in regions of previously low incidence. 5 Year colon cancer survival in the UK (60%) is worse than other Organisation for Economic Co-operation and Development (OECD) countries such as Australia (71%) and Belgium (68%). [2] The epidemiology of the illness is also changing with increasing incidence in younger patients (<50 years) reported in the UK, other western nations and Asia. Younger patients predominantly present with left sided and rectal cancers whilst older patients tend to have right colon cancers. [3]

1.2 Anatomy of the rectum

As the primitive gut elongates, in the third week of gestation it differentiates into the anterior foregut, central mid gut and posterior hind gut. The hind gut encompasses the distal third of transverse colon, descending colon, sigmoid colon, rectum and anal canal above the dentate line. The blood supply to the hind gut is from the inferior mesenteric artery (IMA). During the 5th to 10th week of gestation the protoderm, an invagination of the ectoderm fuses with the hind gut to form the lower third of the rectum. The rectum originates from the from the end of sigmoid colon where the taeniae coli and appendices

epiploic end. [4] The definition of the proximal and distal aspects of the rectum are debated with surgeons considering at the level of the sacral promontory but anatomists considering at the level of the third sacral vertebrae. The distal extent is at the level of the muscular anorectal ring by surgeons but the dentate line as per anatomists. [5] The rectum is divided into three parts: the low rectum (anal verge to 6cm); mid rectum (7-11cm); and upper rectum (12-15cm). It is covered anteriorly and superiorly by peritoneum. The majority of the rectum is extra peritoneal at its posterior aspect and sits on the sacral hollow. The rectum is surrounded by peri-rectal areolar tissue, thicker posteriorly, containing the terminal branches of the IMA. The mesorectum is held in place by suspensory lateral ligaments made up of connective tissue and nerves supplying the rectum which attach it to the pelvic autonomic nerve plexus. The mesorectum does not contain any functionally significant nerves so can be removed as it contains lymphatics that drain the rectum. It also provides a relatively bloodless plane, the “holy plane” as described by Heald. [6] Surgery on the rectums aim to remove this layer intact whilst being careful not to damage the seminal vesicles and posterior vaginal wall in women which is separated by Denonvillier’s fascia. [6] [5]

The blood supply to the hind gut is from the IMA. The IMA travels in the sigmoid mesocolon becoming the superior haemorrhoidal artery when it crosses the left iliac vessels. It bifurcates entering the rectum posteriorly and then running within the submucosa inferiorly to supply the lower rectum and anal canal. The rectum receives its main blood supply from the superior and inferior haemorrhoidal arteries, the latter a branch of the internal pudendal artery, a branch of the internal iliac arteries. Venous drainage of the anorectum is via the middle and inferior haemorrhoidal veins to the internal iliac vein and vena cava. The lymphatic drainage of the upper two-thirds of the rectum is entirely superiorly by the inferior mesenteric nodes, and then to the para-aortic lymph nodes. This

is the reasoning behind a high ligation of the IMA close to its origin to maximise lymph node yield. The lower third of the rectum drains superiorly via the superior and middle haemorrhoidal vessels as well as via the middle haemorrhoidal vessels to the internal iliac lymph nodes. The anal canal is drained by the inferior mesenteric and internal iliac nodes above the dentate line, and by the inferior rectal to the superficial inguinal nodes as well as along the inferior haemorrhoidal artery below the dentate line. Sympathetic innervation of the rectum and left colon is from preganglionic fibres via the lumbar sympathetic nerves arising from L1-L3 which follow the arterial supply from the IMA, and the hypogastric plexus innervating the lower rectum. Parasympathetic innervation of the rectum and anal canal if from the nervi erigentes from S2-4 which follow the sympathetic hypogastric plexus. Pelvic nerves found between the peritoneum and endopelvic fascia and run the risk of injury during rectal surgery which can result in bladder, bowel and sexual dysfunction. [5]

1.3 Risk Factors

Male gender and increasing age have consistently been shown to have strong associations with the incidence of CRC. Colorectal cancer is predominantly sporadic in nature with only 10-20% patients having a positive family history. [7] Migrant populations from lower risk regions soon take on local levels of higher risk. Green leafy vegetables are associated with lower colorectal cancer risk in men, but consumption of fruits was not related to risk in men or women. Vegetables contain micronutrients like carotenoids, folate, ascorbate, bioactive components like phenols, flavonoids, isothiocyanates and indoles, all of which have anticarcinogenic components. Non-digestible fructo-oligosaccharides maybe protective by enhancing beneficial Bifidobacterium and Lactobacillus. [8] A diet high in fibre and increasing dietary fibre have been shown to be protective against colorectal cancer. [9] [10] Lifetime alcohol has been shown to increase the incidence of rectal cancer

more than colonic cancers. [11] Red and processed meats have been shown to increase the risk of colorectal by its effect on bile acid production, formation to carcinogens during the cooking process, and nitrosamine production. An ultra processed diet high in sugar, saturated and total fats have also been implicated. [12, 13] Countries with a high Human Development Index (HDI) have a fourfold incidence of CRCs. Whilst incidence stabilises in the high HDI countries, it is rapidly increasing in low HDI countries due to increased exposure of CRC risk factors. [14]

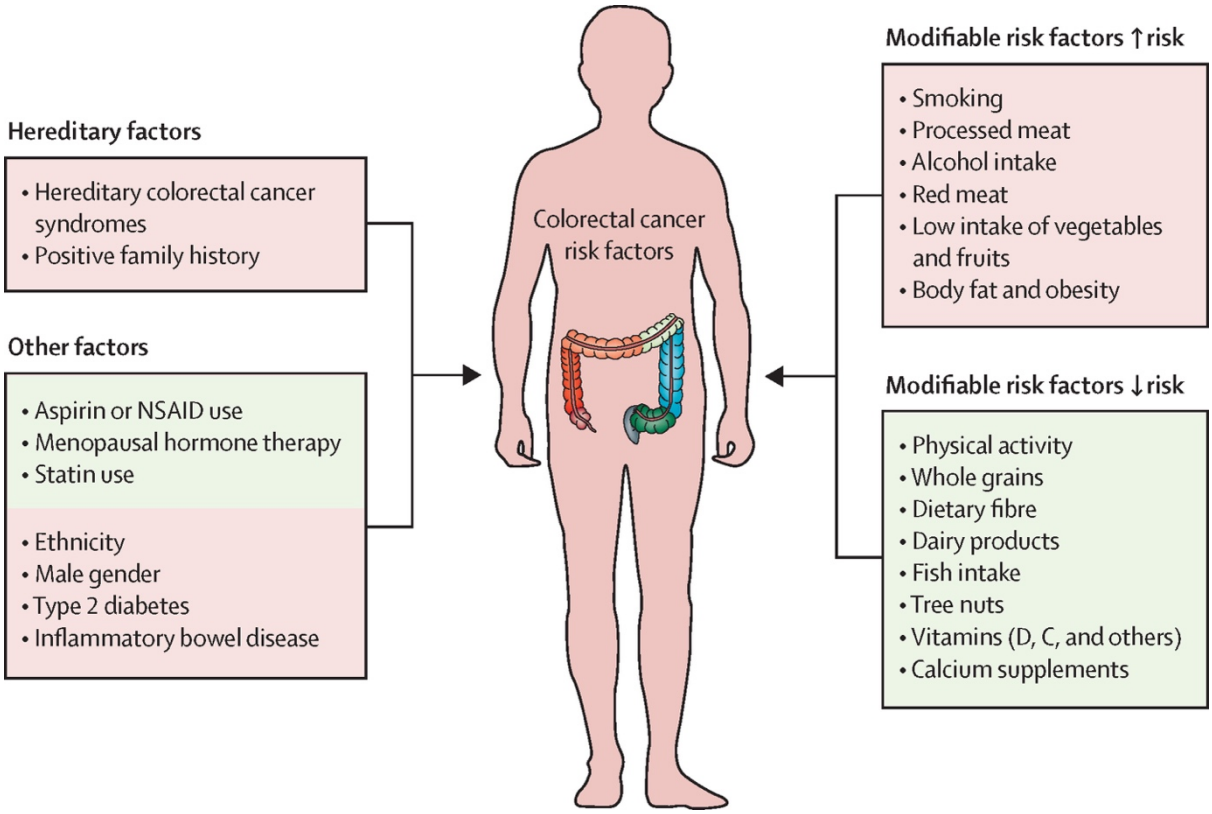
Non dietary risk factors include smoking which has an association with large colorectal adenomas which has the potential to evolve into an adenocarcinoma through the adenoma carcinoma sequence. Chronic inflammation of the GI tract from ulcerative colitis and Crohn's disease increases the risk of CRC. [15] The metabolic syndrome (which includes 3 or more of the following: hypertension; increased waist circumference; hypertriglyceridemia; low levels high density lipoprotein cholesterol; diabetes / hyperglycaemia) has an increased incidence of CRCs in men but not women. [16] Weight gain and heavy alcohol consumption been shown to increase the risk of CRC. [17, 18]

Since 2003, there has been a rising incidence of early onset CRC, nominally referring to patients below 50 years of age, the timepoint at which most colorectal screening programs begin. [19] This has been observed in several high income countries and in the USA follows a strong birth cohort effect with the lowest incidence of CRC in those born in the 1950s. This is now resulting in a higher mortality in the under 55s with CRC. [20, 21] The strong birth cohort effect is indicative of population wide changes in behaviours which increase the risk of cancer such as those detailed above. Antibiotic use has been associated with increased risk of colonic adenoma and CRC via alteration of gut microbiome. [22, 23] CRC progression is mediated by complex interaction between the gut microbiome, inflammation, host genetics and other environmental factors. [24]

1.4 Hereditary CRC

Hereditary CRC can be divided as non-polyposis syndromes which include Lynch syndrome and familial colorectal cancer, and polyposis syndromes. Lynch syndrome is a result of dysfunctional DNA mismatch repair system characterised by expansion or contraction of microsatellite regions in tumour tissue also referred to as microsatellite instability (MSI). These tumours also show a deficiency in mismatch repair proteins on immunohistochemistry. Therefore patients with Lynch syndrome are recommend to undergo colonoscopy every 1-2 years from the age of 20-25 years old due to the accelerated adenoma carcinoma pathway. [7] Polyposis syndromes include three hereditary syndromes (Familial Adenomatous Polyposis (FAP); Attenuated Familial Adenomatous Polyposis (AFAP); MUTYH associated polyposis (MAP)) where germline mutations result in colorectal carcinogenesis manifested by early onset multiple colorectal adenomas, risking earlier development of CRC. FAP caused by germline mutations of the APC gene, is defined by the presence of ≥100 synchronous colorectal adenomas and is inherited in an autosomal dominant manner. AFAP is caused by APC mutations at the far proximal or distal end of the gene, or in certain locations giving an attenuated phenotype with <100 adenomas at presentation, also inherited in an autosomal dominant pattern. MAP has mutated MUTYH involved in DNA oxidative damage repair in multiple genes including APC and KRAS. Polyp and carcinogenesis occurs with germline MUTYH mutations and most commonly found in patients presenting with 20-99 adenomas. [25] In FAP 1-3 yearly colonoscopy from the age of 12-14 is recommended and in MAP annual colonoscopy from 18-20 is recommended under UK guidelines. [26] Risk factors in colorectal cancer are summarised below in figure 1.1

Figure 1.1 Modifiable and non-modifiable risk factors for colorectal cancer. Dekker et al [7]



1.5 Diagnosis and Staging

Patients can present with CRCs with a variety of symptoms and signs, most commonly occult or overt rectal bleeding, changing bowel habit, anaemia or abdominal pain. CRCs are predominantly asymptomatic until advanced disease. As rectal bleeding can present in benign disease, other red flag symptoms described above can be used to identify which patients require colonoscopy. New onset rectal bleeding in patients aged 45 years or older should undergo a colonoscopy. Other red flag symptoms such as weight loss, family history, changes in bowel habit and blood mixed with stool can be helped to identify CRCs in younger patients. [7, 27]

Colonoscopy is the gold standard investigation in the investigation of CRC allowing for lesion visualisation and biopsy. Whilst advanced disease is straight forward, early CRCs

may appear as subtle mucosal lesions, emphasising the importance of good bowel preparation and careful mucosal evaluation. [7] The quality assurance indicator of colonoscopy is further detailed under screening. CT colonography can be used to complete colonic evaluation if colonoscopy is incomplete due to the risk of synchronous colon cancer which can range from 1.1-8.1%. [28] MRI is used for the local staging of rectal cancer and CT scanning for regional staging and to assess the presence of metastatic disease. PET-CT scanning has a role in quantifying the extent of disease particularly if there is dubiety in the presence of metastatic disease. [29] [30, 31]

The evolution of MRI to locally stage rectal cancer has helped to gain local control of disease and reduce the risk of recurrence. The Dutch TME trial demonstrated the low and high risk groups of patients. Low risk patients with superficial tumour can be treated surgically but surgery only for higher risk patients with more advanced tumours which are close to or involving the mesorectal fascia (MRF) which is the circumferential margin of the total mesorectal excision (TME) or involving other pelvic organs will result in incomplete resections and increased rates of local recurrence. [32] MRI is therefore ingrained into international guidelines for the work up of patients with rectal cancer. [29, 33] The MERCURY study group assessing the accuracy of MRF clearance found 92% specificity in margin clearance at the time of surgery. [34] When comparing endoscopic ultrasound (EUS), CT and MRI for T stage sensitivity was 100%, 75% and 72.3% respectively, and N stage sensitivity was 72.2%, 88% and 76.4% respectively. [35] Nodal staging can be difficult on MRI with nodes >9mm having a 93% malignancy risk compared to 50% in nodes 2-5m in size. Irregular border, heterogenous texture and round shape are more in predictive of malignancy than size alone which are considered in international guidelines. The role of EUS is largely to differentiate between T1 and T2 rectal cancers. [29]

Post chemoradiotherapy (CRT) restaging occurs 8-10 weeks following completion of CRT and is recommended. [29] Restaging can show tumour regression from previously involved surrounding organs, MRF margin and is helpful in the assessment of patients who have a (near) complete response who can be managed with organ preservation (watch and wait strategy). MRI has a pooled sensitivity of 40.3% for ypT staging and 19.1% for ypT0 stage (pathological complete response (pCR)), but diffusion weighted imaging (DWI) increases pooled sensitivity of pCR to 83.6%. [36] Endoscopy and digital rectal examination is more accurate at identifying compete tumour response compared to DWI and MRI (area under the curve [AUC] 0.88 vs 0.79). AUC is highest at 0.91 when all four assessment modalities are used, and the recommendation of the International Watch and Wait Database consortium. [37] MRI was more accurate in restaging lymph nodes than at pre-CRT with the absence of mesorectal and extramesorectal nodes being highly predictive of clinical nodal response and lateral node response. [38] [39]

1.6 Screening

The rationale behind colorectal cancer screening is to identify pre-cancerous or pre-clinical lesions in healthy individuals allowing for timely treatment. Screening aims to detect the majority (90%) of CRCs which occur sporadically. [16] Screening and more effective treatment strategies have improved CRC survival in Western nations. [40, 41] Screening aims to interrupt the adenoma carcinoma sequence and treat adenomas occurring via the de novo and serrated pathways. Institution of population based screening using annual faecal occult blood testing has shown to reduce mortality and increase 5 year survival. [42]Latest guidelines recommend testing medium risk individuals over the age of 50. Screening methods include faecal, endoscopic, radiological and blood testing. [41]

Guaiac Faecal occult blood tests (FOBT) identify the presence of haemoglobin in faeces through peroxidase interaction between haem and Guaiac. Due to the requirement of having to repeat the tests on three separate day, dietary constraints and bleeding not being distinguishable between the upper and lower GI tract, this has been superseded by faecal immunochemical testing (FIT). FIT testing is not dependent on dietary restrictions, requires fewer samples and identifies lower GI bleeding. FIT also has a greater sensitivity that FOBT in identifying precancerous lesions and CRC. [41, 43, 44] FIT provides quantitative measurements but an optimal cut off has not been identified. The ideal cut off is largely based on endoscopic capacity, incidence and prevalence of CRC, and expected adherence to the screening program. Quantitative FIT testing has thus become part of the CRC screening program in the European, American, Western Pacific and East Asian counties. [45] In the UK, FIT testing is the method of CRC screening in individuals aged 54-74 years old in England and 50-74 in Scotland and Wales. The use of faecal DNA detection kits which combines qualitative colorectal neoplasia associated DNA markers with occult haemoglobin biomarkers associated with CRC. Faecal DNA testing has not taken off due to being less cost-effective than annual FIT testing and in some countries more expensive than colonoscopy. [41]

Flexible sigmoidoscopy (FS) is used as a screening tool for CRC (but not in the UK or North America). It allows limited bowel preparation, quicker examination, lower complications, minimal discomfort, biopsies, polyp retrieval and is cheaper. As it only checks the distal 60cm of colon and rectum, it does not prevent proximal CRC. FS can reduce CRC incidence by 32% and mortality by 50%. [46] Colonoscopy is the only test capable of visualising all the colon and being able to remove precancerous lesions. It has

been shown to reduce CRC specific mortality by 80% in the distal colon and 60% in the proximal colon. It can be used as a screening test primarily or following on from positive FIT testing. [41] The effectiveness of colonoscopy is dependent on accurate detection and removal of colonic polyps. Quality control measures for this include: ≥25% adenoma detection rate; ≥95% caecal intubation rate; >6minute withdrawal time; and good quality bowel preparation. [47] Capsule endoscopy, whilst having fewer procedural and sedation related complications, has poorer sensitivity and specificity compared to colonoscopy. [41]

Computed tomographic colonography (CTC) is a newer screening test with reduced sedation and procedure related risks than colonoscopy, therefore a suitable alternative to those who cannot undergo a colonoscopy. It identifies 90% of polyps >10mm and 70-80% of polyps between 6-9mm. It has a similar diagnostic yield for advanced neoplasia to colonoscopy indicating safe use in population based screening. [48] Disadvantages include: radiation dose (especially if having to be repeated); requirement for bowel preparation; not detecting smaller polyps; flat adenomas; serrated adenomas (with greater malignant potential); requirement for colonoscopy if positive; and a higher overall cost. [41]

1.7 Carcinogenesis Pathway

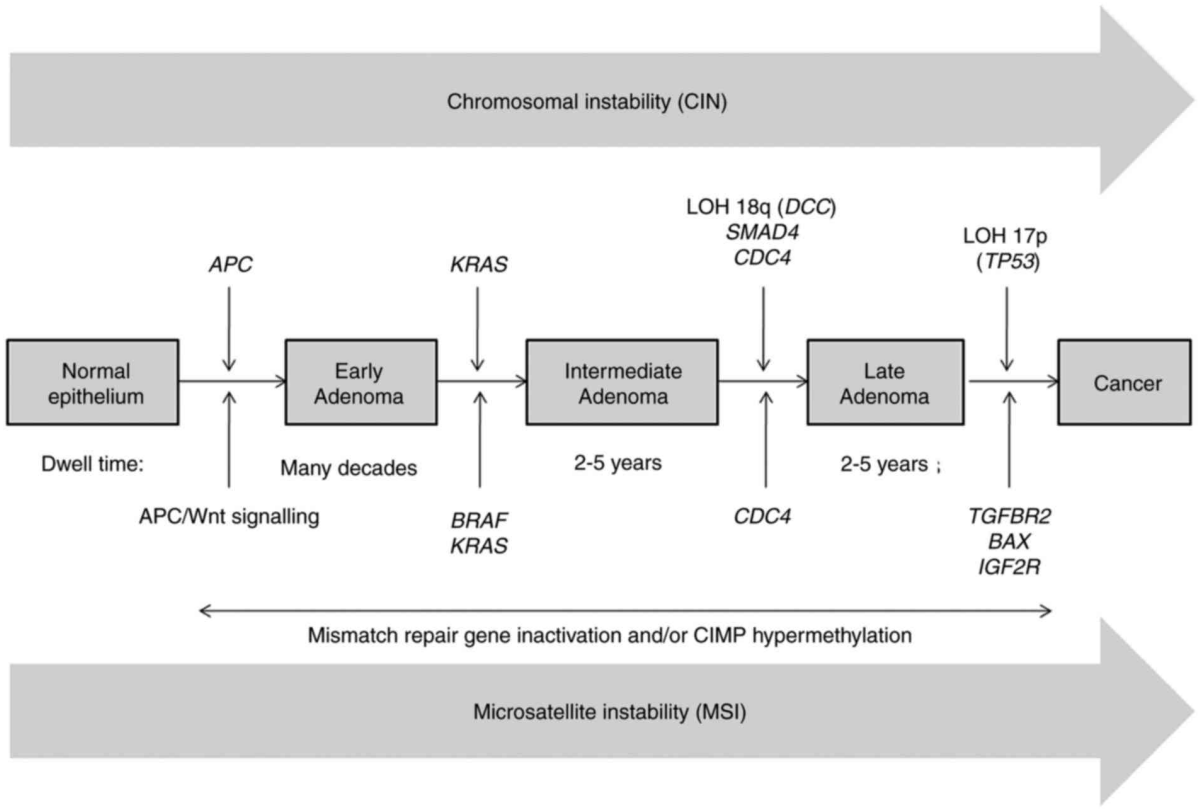
CRCs occur predominantly through two district molecular pathways summarised in figure 1.2 Approximately 70% occur through better understood chromosomal instability pathways (adenoma carcinoma sequence). Over the last two decades the “serrated neoplasia pathways” responsible for approximately 30% of CRCs is being better understood with improved endoscopic and pathological assessment of serrated polyps and cancers. [49]

The multistep carcinoma sequence described by Fearon and Vogelstein starts with APC inactivation, followed by KRAS mutations in adenoma stage. Deletion of chromosome 18q and inactivation of tumour suppressor gene TP53 on chromosome 17p occur during the transition to malignancy. [50] [51] [52] [53] Allelic loss at chromosomal regions results in mutations of tumour suppressor genes: APC on chromosome 5q; TP53 on chromosome 17q; DCC netrin 1 receptor (DCC); SMAD family (SMAD 2 and SMAD 4) on chromosome 18q. Gains in chromosomes 7 and chromosomal arms 1q, 8q, 12q, 13q and 20q have oncogene potential, favouring growth and survival. [54] These changes which result in gain or loss of function of tumour associated genes allows mutated cells to have growth and survival advantages which results in the progression of normal cells to cancer cells. [55]

Microsatellite instability (MSI) pathway includes 2 subtypes. These are germline mutations in the MMR gene followed by a second hit to the wild type gene copy from point mutation, loss of heterozygosity, or methylation. These germline mutations lead to Lynch syndrome which form 20% of MSI CRCs. The remainder are non-familial MSI CRCs are from epigenetic inactivation of the MLH 1 gene which occur on a background of CIMP resulting in hypermethylation of promoter genes showing CIMP and BRAFV600E hotspot mutations. [56]

The serrated neoplasia pathway which makes 10-20% of CRCs includes the traditional serrated and sessile serrated pathways. The process starts with mutation of BRAF or KRAS gens but progress by methylation of tumour suppressor genes CpG island methylator phenotype (CIMP). This can result in microsatellite stable and unstable tumours. [7] [57]

Figure1.2 shows the stepwise adenoma carcinoma sequence. Taken from Nguyen et al [58]



1.8 CIMP pathway

The molecular profiles described by Jass are useful in characterising these pathways. The main differentiators being DNA microsatellite instability (MSI) stratified as: MSI-High (MSI-H); MSI low (MSI-L); MS Stable (MSS) and cpG island methylator phenotype (CIMP) stratified as: CIMP-high; CIMP-low; and CIMP-negative (CIMP-neg). Thus, five molecular subtypes have been described:

Type 1 (CIMP-high / MSI-H/ BRAF mutation)

Type 2 (CIMP-high/ MSI-L or MSS/ BRAF mutation)

Type 3 (CIMP-low/ MSI-L or MSS/ KRAS mutation)

Type 4 (CIMP-neg/ MSS)

Type 5 (CIMP-neg/ MSI-H) or Lynch Syndrome

Type 1 and 2 CRC have serrated polyps as the precursor lesions. Type 4 and 5 CRCs are from polyps following the adenoma carcinoma pathway. Type 3 CRCs can occur via either type of polyp / pathway. [59]. Sessile serrated adenomas (SSA) typically occur proximally, have a characteristic crypt disturbance and have BRAF mutation. SSAs have an increased risk of progression through their genetic abnormalities. Traditional serrated adenomas favour the left colon with tubulovillous architecture, eosinophilic cytoplasm and often have KRAS mutation. [49]

Adenomas occur because of alteration of normal mechanisms which regulate DNA repair and cell proliferation. Intestinal mucosa undergoes constant epithelial cell renewal due to the loss of mucosal surface cells. Cell proliferation occurs at the crypt base. Adenomas form as cells with mutations advance to the lumen by terminal differentiation and apoptotic disruption. These adenomatous polyps can grow, become more dysplastic in nature and can become invasive. Mutations typically occur the adenomatous polyposis coli (APC) gene followed by RAS inactivation or functional loss of TP53 responsible for tumour suppression, or BRAF oncogenes give rise to traditional adenomas and serrated adenomas respectively. [7, 60]

1.9 Consensus molecular subtypes

The Colorectal Cancer Subtyping Consortium described the four Consensus Molecular Subtypes (CMS) based on the analysis of 4000 Stage II and III CRCs and summarised below in figure 1.3. CMS 1 subtype tumours are MSI, immune active, hypermutated, BRAF mutated and predominantly affect the proximal colon. CMS 2 subtype tumours are microsatellite stable, have high CIN, strong WNT/MYC pathway activation, EGFR

amplification or expression, have mutated TP53 and predominantly affect the distal colon and rectum. CMS 3 subtype tumours have low CIN, moderate WNT/MYC pathway activation, mutated KRAS, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha gene (PIK3CA), and insulin-like growth factor binding protein 2 (IGFBP2) overexpression. CMS 4 subtype are mesenchymal type CIN / MSI heterogenous tumours with transforming growth factor beta (TGF-β) activation and have neurogenic locus notch homolog protein 3 (NOTCH3) and vascular endothelial growth factor receptor 2 (VEGFR2) overexpression. CMS 3 and CMS 4 subtypes do not have anatomical preponderance. Survival is highest in CMS 2 and poorest in CMS 4, with intermediate survival in CMS 1 and CMS 2 tumours. [61] [62]

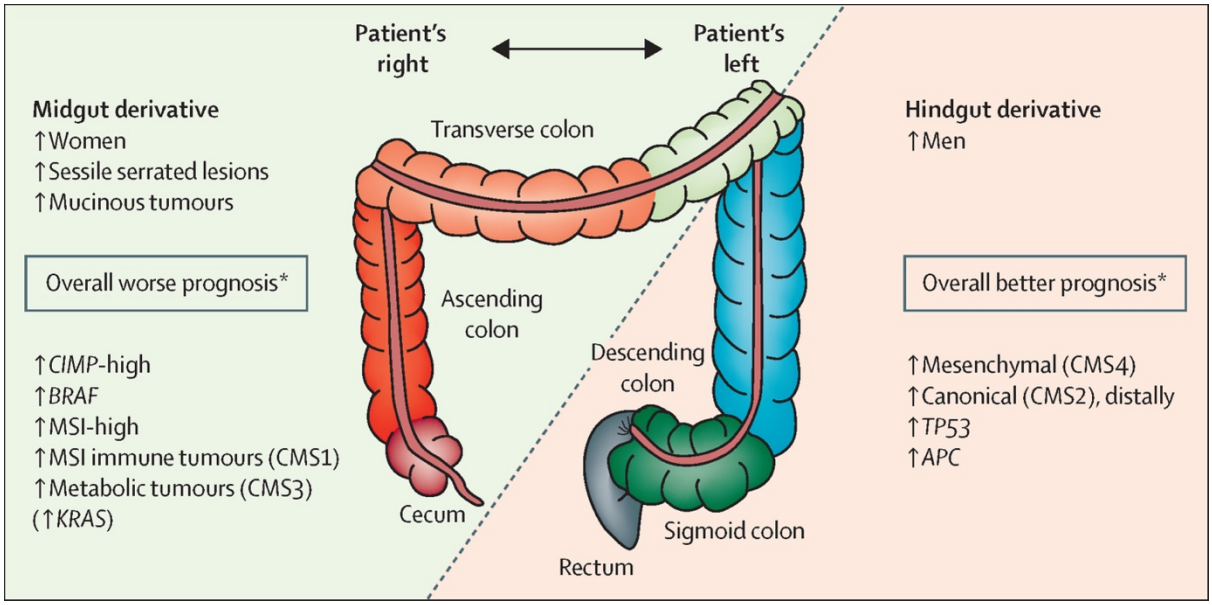
Figure 1.3 Characteristics of each SMC subtype taken from Guinney et al. (%), percentage of samples. [61]

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

1.10 Right and left sided disease

Colorectal cancers differ according to location in their embryological, molecular, biological and prognostic features. Colorectal cancers are split into right and left sided cancers summarised below in Figure 1.4.

Figure 1.4 showing the differences between right and left sided colon, and rectal cancers taken from Dekker et al. * Prognosis being applies to metastatic disease and response to anti-EGFR and anti-VEGF therapies. Right sided cancers have a higher incidence of MSI high cancers. Previously this had been associated with poorer prognosis with metastatic disease but immunotherapy has provided new treatment avenues [7]



The incidence of mucinous and signet cell tumours is higher in the proximal colon compared to the distal colon or rectum (approximately 45% vs 20%).[63]. Patients with FAP develop distal colon cancers (approximately 60% left sided and 25% rectal) as opposed to patients with HNPCC who develop predominantly proximal tumours (approximately 55% right sided and 15% rectal). [64] [65]. The three main types of epigenetic instability in colorectal cancers are CIN, MSI and CIMP. Sporadic CIN tumours can occur anywhere in the large bowel from adenomas. KRAS oncogene mutations in CIN tumours causes resistance to anti-EGFR therapy. [66] [67] [68] Sporadic MSI tumours

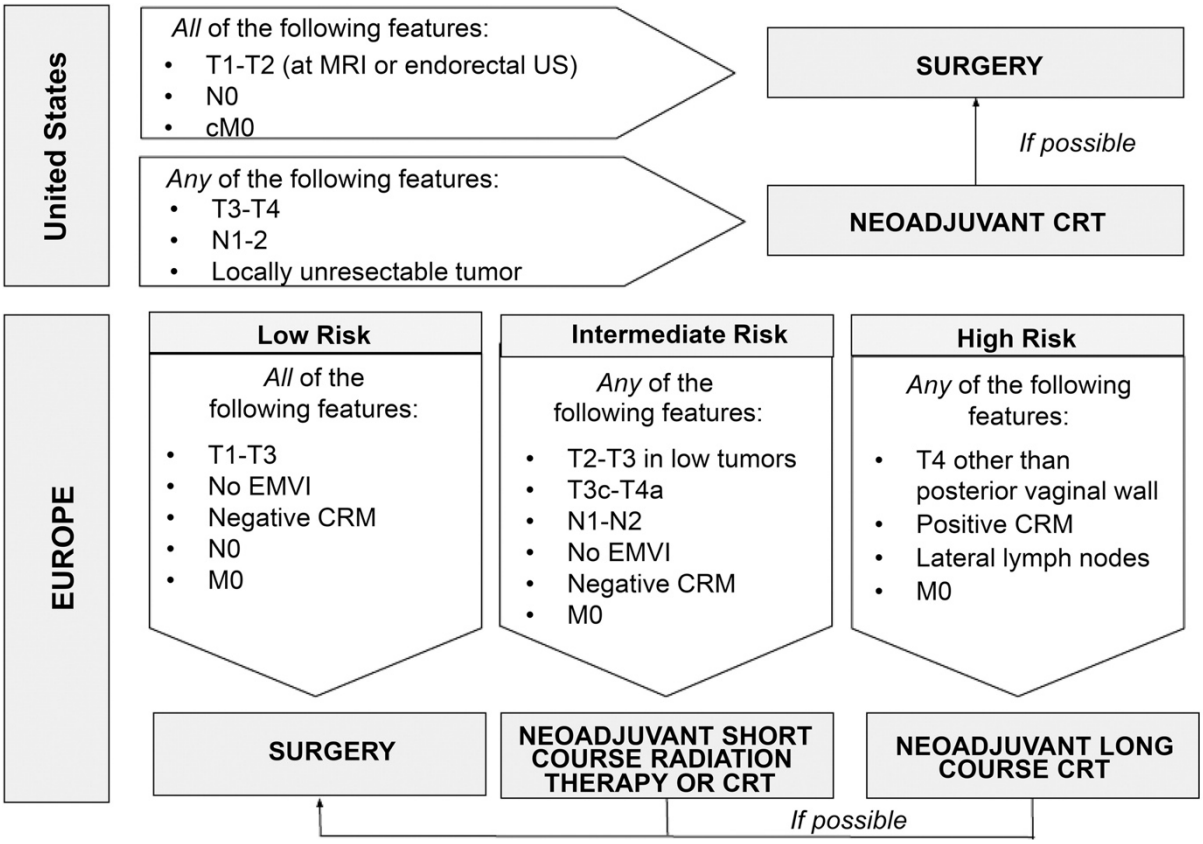
mostly occur in the proximal colon, are often mucinous and rarely occur in the rectum. These tumours with BRAF oncogenes cause MLH1 hypermethylation resulting in anti-EGFR therapy resistance. [69] [70] Sporadic CIMP tumours occur within sporadic sessile serrated adenocarcinomas of the proximal colon having MHH 1 hypermethylation and in traditional serrated adenocarcinomas of the distal colon and rectum having MGMT methylation . [65] In a study of over 1400 patients with stage I-IV colorectal cancer the incidence of MSI high, BRAF mutations and CIMP high tumours decreased in proximal colon to the rectum. [71] The Pan European Trial Adjuvant Colon Cancer 3 study found during molecular analysis that proximal tumours were more often MSI, hypermutated, BRAF mutated, had a serrated signature and has dense infiltration of tumour related lymphocytes. Distal tumours were CIN, HER 1and 2 amplified, had EGFR signalling and were not BRAF like. [72]

1.11 Management

The investigation and management of rectal cancer is by a multidisciplinary team (MDT) consisting of surgeons, oncologists, radiologists, pathologists and allied health professionals guiding patients through their treatment journey whilst maintaining the highest oncological standards. MDT presentations (or tumour boards) of imaging, evidence based oncology, surgical recommendations and pathological assessment of surgical specimens are paramount in providing high quality cancer care. Surgical management should be guided by patient and tumour factors whilst trying to main function and survival by means of minimising recurrence. Clinical assessment of rectal cancers by colorectal surgeons are key to understanding tumour location, mobility, proximity to anal sphincter and pelvic floor assessment. This will help guide management between formal TME resection, local excision or in organ preservation in some patients. [73]

Early rectal cancers (T1) are amenable to local treatment by transanal endoscopic surgery but will not have a lymphadenectomy. Recommendations are that this is only used for clinical selected T1 disease. [74] Resection specimens need pathological assessment for deep and lateral margins, as well as high risk features such as: depth of submucosal invasion; differentiation; lymphatic invasion; and tumour budding which may prompt recommendation for radical resection. [7, 75-77] Local excision has been used in T2N0 cancers (ACOSOG Z6041 trial) [78]. GRECCAR 2 trial compared local excision with TME resection in patients who have had a good response to CRT with small residual tumours (≤2cm) showed no differences in oncological outcomes.{Rullier, 2020 #3269} This does provide an option for patient unwilling or unable to undergo TME resection. The STAR-TREC trial aims to investigate cancers up T3b comparing: standard of care (TME); organ preservation followed by short course radiotherapy; or organ preservation followed by long course chemoradiotherapy. [79] Management of rectal cancer is summarised in figure 1.5.

Figure 1.5 Schematic summary of the management of rectal cancer from American and European guidelines, taken from Hortvat et al [80]



Surgical resection for rectal cancer can be challenging due to surgical access to the pelvis and pelvic anatomy. The aim of surgical resection is to remove the rectum with surrounding mesorectum as to obtain an R0 resection with micro and macroscopic resection margins. [7] Total mesorectal excision is the gold standard oncological resection along embryological fascial planes, which includes lymphatics, to minimise local recurrence as described by Heald. [81] Tumour proximity and involvement of the anal sphincter which can affect continence may necessitate an abdominoperineal resection. If this is not the case a colorectal or coloanal anastomosis can be performed which may require a defunctioing stoma to reduce the morbidity associated with an anastomotic leak. [7, 82]. Surgery can be performed by open, laparoscopic, hand assisted, robotic and transanal approaches. Laparoscopic surgery for rectal cancer has been shown to have post operative benefit and has longer term outcomes similar to open surgery with low rates of

conversion. [83, 84] Robotic assisted surgery has also been shown to have similar oncological outcomes to laparoscopic surgery. [85]

1.12 Neoadjuvant therapy

Despite surgery alone being adequate for early stage cancers, locally advanced rectal cancer (LARC) carried local recurrences of up to 16% in stage II and 29% in stage III disease historically. [86] Local recurrence can be reduced to approximately 5% with neoadjuvant therapy, and is therefore recommended following MRI staging. [87] [74] [88] Neoadjuvant therapy for LARC results in varying grades of tumour regression in 65%, pCR in 14% and no tumour regression in 20% of patients. [89] [90] [91] Overall survival in complete responders can vary between 73% and 90% but mortality can be as much as 30% from distant disease in those who have a poor response. [92] [93, 94]

The two forms of neoadjuvant radiotherapy are: short course radiotherapy (SCRT) giving 25Gy in 5 fractions; and long course chemoradiotherapy (LCRT) giving 45-50.4Gy in 25-38 fractions with concurrent fluoropyrimidine or oxaliplatin chemotherapy as radiosensitisers. Fluoropyrimidine like 5-FU or capecitabine prevents nucleoside formation, and oxaliplatin works by forming DNA-platinum adducts, essential for tumour division. [74] [88]. [95]

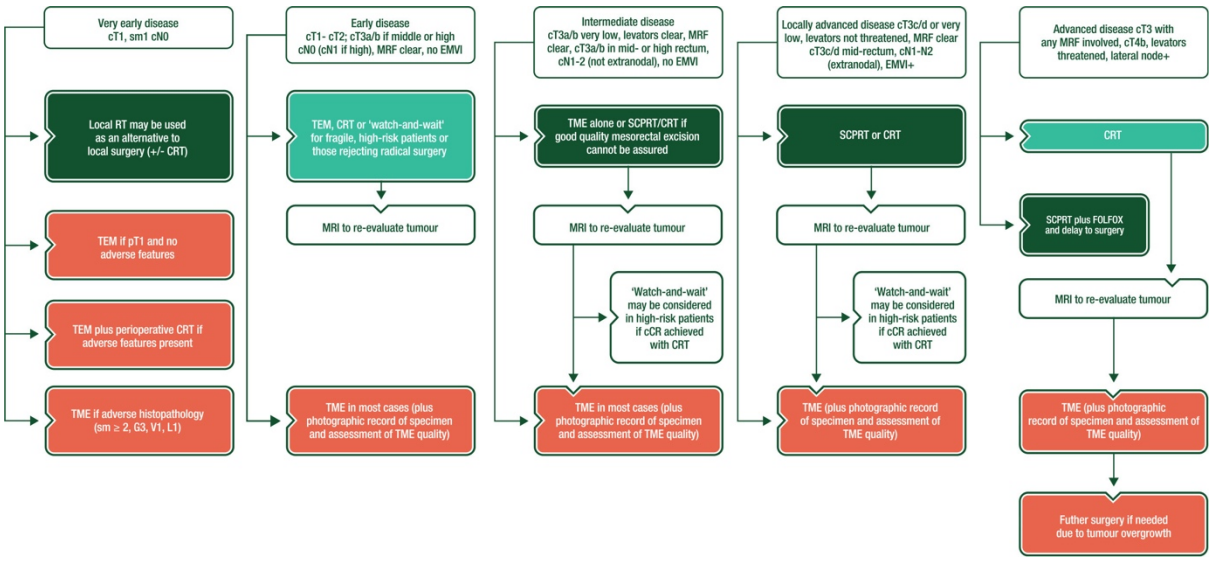
The landmark trials in neoadjuvant management of LARC are detailed in table 1.1.

The Swedish Rectal study demonstrated neoadjuvant SCRT followed by surgery has reduced rates of local recurrence and improved DFS/OS.[96] [97] The Dutch TME trial validated improved local control but found no difference in OS/DFS. [32] SCRT has traditionally be followed by surgery the following week which inferior downstaging outcomes compared LCRT but similar local disease control as per the Polish and Tans-Tasman studies. [89] [98] The AC-10-94 trial showed neoadjuvant LCRT was more

effective for local control than adjuvant LCRT but there was no difference in OS. [99] Pre-Op SCRT was shown to have improved local control compared to selective post-op LCRT in cases of R1 resection. [100] NSABPR-04 also showed improved DFS / OS with pre-op LCRT compared to post-op LCRT. [101] Delay between LCRT and surgery showed surgery at 6 weeks allowed for greater tumour downstaging and increased likelihood for sphincter preservation up to 6 years. [101]

European Society of Medical Oncology (ESMO) guidelines for rectal cancer advise the use of short course RT or long course CRT in early disease as an alternative to surgery or with a view to achieve complete response and enter Watch and Wait protocol. Intermediate disease can be treated with TME resection or have neoadjuvant treatment should there be risk that good quality mesorectal excision cannot be achieved. For locally advanced disease the recommendation is neoadjuvant therapy with short course RT or CRT prior to reassessment / surgery. CRT or short course RT with FOLFOX and delay is recommended for advanced disease. In summary there is no clear T or N substage which would indicate SC RT or CRT but if CRM or R0 resection is threatened the recommendation is CRT. Neoadjuvant chemotherapy alone is not recommended for the treatment of localised non-metastatic disease out with clinical trials due to the lack of long term oncological data for advanced disease. [102] This is summarised in the figure 1.6 below.

Figure 1.6 Detailing treatment recommendation from ESMO guidelines 2017 Glynne-Jones et al. cCR, clinical complete response; CRT, chemoradiotherapy; EMVI, extramural vascular invasion; FOLFOX, leucovorin/fluorouracil/oxaliplatin; MRF, mesorectal fascia; MRI, magnetic resonance imaging; RT, radiotherapy; SCPRT, short-course preoperative radiotherapy; TEM, transanal endoscopic microsurgery; TME, total mesorectal excision



In the UK, National Institute of Clinical Excellence (NICE) does not recommend radiotherapy in early rectal cancer outwit clinic trials. [88] American guidelines advise that neoadjuvant therapy should be given to patients with clinical stage II and stage III disease and that this is tailored to the individual patient's risk following MDT discussion. [103]

Whist the ESMO and NICE guidelines do not comment on induction or consolidative chemotherapy prior to or following CRT and before resection, this Total Neoadjuvant Therapy (TNT) approach is an acceptable approach as per the National Comprehensive Cancer Network (NCCN) guidelines from the USA. [104] Two systematic reviews have shown TNT increasing the rate of pCR and improve disease free survival, with one of these also improving overall survival and risk of distant metastasis.

[105, 106] Induction chemotherapy has been shown to be better tolerated with lower acute toxicity and improved compliance over adjuvant chemotherapy in the Spanish GCR-3 study. [107] The CAO / ARO / AIO-12 trial has shown consolidation chemotherapy is associated with higher rates of pCR and with no differences found in disease free or locoregional survival. [108] At present there is no consensus over the superiority of induction vs consolidation chemotherapy and the subject of ongoing trials. In the UK practice is shifting to a TNT approach for locally advanced and advanced rectal cancer.

1.13 Immunotherapy

Immunotherapy targeting immune checkpoints has proven effective in various tumour types including melanoma, where antibodies to T-cell inhibitory molecules enhance effector T cell function and improves progression free survival (PFS) and overall survival (OS) [109, 110]. T-cell regulatory receptor Programmed Cell Death – 1 (PD-1) and its ligand (PD-L1) are expressed in most cancers [111] where together they dampen anti-tumour T-cell responses. Tumours thought to be responsive to immune checkpoint inhibition include those with high mutational load and neoantigen burden [112], or evidence of an immune active tumour microenvironment (CD3/CD8+ infiltrates) [113, 114]. PD-1/PD-L1 expression and presence of T cells within the TME are considered useful indicators anti PD-1 therapy may be effective [113, 114].

Out with the mismatch repair deficient (dMMR) subtype, most CRCs lack presence of T cells and PD1/ PD-L1 expression within the TME, limiting use of anti-PD-1 therapy [114-116] [117]. PD-1 is poorly expressed in CRC (13%) compared with melanomas (53%) [118]. In dMMR CRC, phase II studies have already reported improved PFS and OS with anti-PD-1 therapy [119, 120]. Defining responsiveness to immunotherapy in CRC is therefore linked to molecular subtype and potentially, measures of tumour immunogenicity.

dMMR tumours make up 25%, 12% and 4 % of stage II, III and IV CRCs respectively and the minority of rectal cancers (10%) [121]. Consequently, anti-PD-1 therapy applies to a tiny subset of all CRCs. Strategies to enhance immunogenicity in dMMR CRC may broaden use of immunotherapy in early and late stage disease. Theoretically, radiotherapy (RT) and cytotoxic chemotherapy (CT) induce cellular damage [122] resulting in tumour antigen exposure and potential generation of T cell responses. Such treatments may synergize with immunotherapies, to enhance response in the neoadjuvant setting with potential optimization of local and systemic control.

Immunotherapy in addition to neoadjuvant therapy shows significant promise in enhancing anti-tumour immune mechanisms. Immune checkpoint inhibitors (ICIs) which block negative regulators of the immune system such as cytotoxic T lymphocyte associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), and programmed cell death-ligand 1 (PD-L1) have been effective in the treatment of solid cancers. [110, 123, 124] The NICHE study of early colon cancers with ipilimumab (Anti-CLTA-4) and nivolumab (Anti-PD-L1) showed pathological response in all patients with dMMR and 27% of pMMR tumours in early colon cancer. In pMMR tumours, CD8+PD-1 T cell infiltration was predictive of response. [125] The likelihood of MSI high cancers is higher colon in the colon than rectum and that MMR status predicts the likelihood of response to immune checkpoint inhibition. [119] Anti-PD-1 antibody in combination with radiation of melanoma and breast carcinoma xenografts increased the proportion of tumour antigen complexes, major histocompatibility complex molecules, enhanced lymph node cross-presentation, and increased T cell tumour infiltration during in vivo studies. [126] The aim of immune therapies in MSS tumours is to overcome an immune cold tumour microenvironment. There are several ongoing studies assessing the combinations of CRT and ICIs In LARC (VOLTAGE; NRG-GI0002; AVANA; AVERECTAL). [127]

Table 1.1 Summary of the major trials for neoadjuvant therapy for rectal cancer.

NSABP R0112	1988	Post op RT 46/47Gy vs TME only vs adjuvant chemotherapy – 5-FU; semustine; vincristine	RT reduced local recurrence compared to TME alone. Chemotherapy improved DFS and OS compared to TME alone.
NCCTG	1991	Adjuvant RT 45-50.4Gy vs adjuvant RT with FU and semustine	Chemotherapy reduced recurrence, distant metastases, cancer specific and overall death
Swedish Rectal Trial	1996	Pre-op SCRT 25Gy vs TME	SCRT reduced local recurrence; improved OS & DFS
Dutch TME trial	2001	SCRT & TME vs TME	SCRT gives better local control but no difference in OS or DFS
A10-94 Trial	2004	Pre-op vs Post Op LCRT	Pre-op LCRT gives better 5 year local control persisting till 10 years but no difference in OS
FFCD9203 EOTRC	2006	SCRT vs LCRT	LCRT gives better local control but no difference in OS
Polish Trial	2006	SCRT & TME within 7 days vs LCRT & TME with delay	No significant difference in local control / OS
NSABP R-03	2009	Pre-op vs post-op LCRT	Pre-op LCRT improved DFS and trended to improve OS
MRC C-07 & NCIC-CTG C016	2009	Pre-op SCRT vs post of LCRT in R1 resections	Pre-op SCRT reduced local recurrence
TROG-01.04	2012	SCRT vs LCRT	No significant difference in local control / OS
NSABP R-04	2015	LCRT with 4 chemo options	Capecitabine non-inferior to IV 5-FU. Oxaliplatin (CAPOX / FOLFOX) adds toxicity without benefit

Timing trial	2015	LCRT & TME 5-6 weeks vs LCRT & consolidation mFOLFOX-6 (if good response at week 4) & TME 2-3 weeks after	mFOLOFOX post LCRT and extending time interval increased pCR
Polish 2 Trial	2016	SCRT & FOLFOX & TME vs LCRT & 5-FU / LV for cT3/cT4	No significant difference in R0/pCR/OS/DFS
Stockholm III	2017	SCRT with immediate TME vs SCRT with delayed TME vs LCRT & TME	No difference in local control or metastases. SCRT with reduced surgical complications vs no delay
AIO-12 trial	2019	Induction FOLFOX & LCRT, TME vs LCRT & consolidation FOLFOX, TME (LCRT with CAPOX)	higher pCR, improved compliance, lower G3/G4 toxicity with consolidation chemotherapy
OPRA	2020	Induction FOLFOX or CAPOX & LCRT vs LCRT & consolidation FOLFOX or CAPOX vs historic control	Organ preservation 43% induction vs 58% consolidation
PRODIGE-23	2020	FOLFIRI & LCRT & TME & 3m adjuvant chemo vs LCRT & delayed TME & 6m adjuvant chemo cT3/4	DFS and MFS better with arm B
RAPIDO	2020	SCRT & CAPOX & TMS vs LCRT & delayed TME	Reduced disease related treatment failure & distant metastases, improved pCR

1.14 Clinical complete response

Some patients can have significant tumour cell death from chemotherapy and radiotherapy to the point they can have complete regression of the primary tumour called pathological complete response (pCR). This has therefore raised the option of avoiding surgery in these patients, which can be potentially unnecessary or harmful. Habr-Gama et al have led assessment of tumour response following neoadjuvant therapy and before surgery to identify patients who have no residual tumour, termed complete clinical response (cCR). These patients were offered a watch and wait strategy with strict surveillance in order to avoid surgery. [128] This group of patients were shown to have similar longer term oncological outcomes compared to patients who underwent surgical resection and had a 95% colostomy free survival and good continence. [129, 130] This has been reproducible across multiple dedicated centres and from data from the International Watch & Wait Database. [131, 132] Patients with complete clinical response can develop local recurrence in up to 25% of cases. This is mainly endoluminal recurrence and amenable to salvage TME resection with no compromise to long term oncological outcomes despite having delayed surgery. Patients who have a complete clinical response and are managed by watch and wait pathway require significant patient compliance with follow up by serial digital examination, endoscopic and MRI evaluation. As such this has yet to be become the widely implemented strategy in managing these patients. [73]

1.15 Proposed watch and wait protocols

Standard CRT regimens had rates of pCR and cCR between 15-30% but CRT with consolidation chemotherapy pCR and cCR rates increased to 30-50%. [133] [134] [135] Habr-Gama et al describe a three pillared assessment of clinical response. First is by digital rectal examination to assess subtle surface changes and ensure there is no ulceration,

palpable mass or stenosis. Endoscopic assessment is the second pillar assessing for tumour or scar appearance, use of advanced imaged such as narrowband imaging, and allowing retroflexion to fully appreciate tumour location to dentate line. The scar is often white with no ulceration of the rectal wall, mucosal abnormalities or stenosis. Whilst there maybe telangiectasia, the presence of irregular areas of redness should be classed as suspicious. The third pillar is radiological assessment of the rectum, mesorectum and pelvis. MRI tumour regression grade (mrTRG) is a proposed classification of tumour response similar to pathological tumour regression grade. T2 Diffusion weighted image sequences give further functional information about tumour repones and can indicate the presence of cancer. [128] [136]

Assessment of response to CRT is very much time dependent with the majority of patients exhibiting response early or immediately after completing CRT. Tumours with poor response immediately following CRT or at 6 weeks are unlikely to develop further significant response thereafter. [137] Due to the risk of tumour regrowth the early assessment is required to ensure regrowth is not mistaken for near-complete ongoing response. [138] Most cCR occurs by six months but if there is still incomplete response by 24-26 weeks following completion of CRT, surgical resection is preferred. [139, 140] Local regrowth occurs in 25-30% patients managed nonoperatively with cCR and most often occur within the first 2-3 years after completion of CRT. [141] The International Watch and Wait Database (IWWD) suggest surveillance protocols have more intense follow during these years to bear this in mind.

National Comprehensive Cancer Network (NCCN) guidelines for watch and wait surveillance patients following cCR is similar to the OPRA study surveillance protocol. This involves digital rectal examination, flexible sigmoidoscopy, and CEA every 4 months for the first 2 years, followed by every 6 months from years 3 to 5. Patients should also have an MRI every 6 months for the first 2 years followed by annual MRI from years 3 to

5, annual thorax abdomen pelvis CT scans for 5 years, and colonoscopies at year 1 and 5. [142, 143]

1.16 Markers of response

1.16.1 Pathological

Pathological response to neoadjuvant CRT (nCRT) can be quantified by tumour regression grade (TRG) and widely used to compare across different study populations. Following radiotherapy tissues develop characteristic necrosis and fibrosis, distinguishable from tumour cells. Tumour regression of the primary tumour can be semi-quantitively determined by the amount of viable tumour in relation to the amount of fibrosis present. This can range from no viable remaining tumour to no evidence of treatment response. [144] [145] [146] The most common TRGs (used to describe treatment response summarised in table 1.2 are by Dworak, Wheeler, Mandard and Ryan. [147] [148] [149] [150]

Table 1.2 Summary of the commonly used pathological tumour regression grades adapted from Ryan et al [151]

Author	Grade	Description
Dworak et al	0	No regression
	1	Dominant tumour mass with obvious fibrous and/or vasculopathy
	2	Dominantly fibrous changes with few tumour cells or groups
	3	Very few tumour cells in fibrotic tissue with or without mucous substance

	4	No tumour cells, only fibrotic mass. Total regression
Mandard et al	1	Complete regression, absence of residual cancer
	2	Presence of rare residual cancer cells scattered through fibrosis
	3	Increased number of cancer cells but fibrosis still predominates
	4	Residual cancer outgrowing fibrosis
	5	Absence of regressive change
Wheeler et al.	1	Sterilization or only microscopic foci of adenocarcinoma remaining, with marked fibrosis
	2	Marked fibrosis but macroscopic disease present
	3	Little or no fibrosis, with abundant macroscopic disease
Ryan et al.	1	Complete regression or only the presence of rare cancer cells
	2	Increased number of cancer cells but fibrosis still predominates
	3	Absence of regressive change or residual cancer out growing fibrosis

1.16.2 MRI

MRI scanning is widely used in the pre-treatment rectal cancer staging and for the quantification of post nCRT response. A limitation of MRI is differentiating between residual tumour and fibrosis, desmoplastic reaction, oedema and inflammation. [151] Most studies have radiologists comparing low and intermediate signal with fibrosis exhibiting the former and tumour the latter. [152] The MERCURY group has published a tumour regression grade based on markers of favourable response (detailed in table 1.3).

Table 1.3 Tumour regression grade following nCRT based on T2 weighted imaging by the MERCURY group. [153]

Grade	Description
1	Absence of any tumour signal
2	Predominance of fibrosis with minimal residual intermediate tumour signal
3	Mixed areas of low-signal fibrosis and intermediate signal intensity present but without predominance of tumour signal
4	Predominantly tumour signal intensity with minimal fibrotic low signal intensity
5	No fibrosis evident; tumour signal visible only

1.16.3 Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a tumour marker in colorectal cancer and recommended to be checked pre-operatively by the NCCN, American Society of Clinical Oncology and European Group on Tumour Markers. Elevated CEA levels are associated with metazoic or recurrent disease and used in addition to imaging investigations as part of

post-operative surveillance. CEA will normalise in 70% of patients undergoing surgery by 6 weeks. Patients with an elevated pre-operative CEA had a 7.4% reduction in 3 year recurrence free survival (RFS) and those with a persistently elevated CEA had a 14.9% lower 3 year RFS. [154] Lower baseline CEA and smaller tumours have been associated with pathological complete response to neoadjuvant chemoradiation, and higher baseline CEA (>5ng/ml) has been associated with poor treatment response. [155, 156]

1.16.4 NAR score

The neoadjuvant rectal (NAR) score was developed shorter term surrogate endpoint for disease free survival (DFS) and overall survival (OS) allowing for more rapid determination of the success or failure of experimental interventions. Score calculation is based on commonly collected clinicopathological data shown in figure 1.7. NAR score has been validated in independent datasets and is a greater predictor of OS than pCR. George et al stratified NAR score as low (<8), intermediate (8-16) and high (>16) with 5 year OS values of 92%, 89% and 68% respectively. [157]

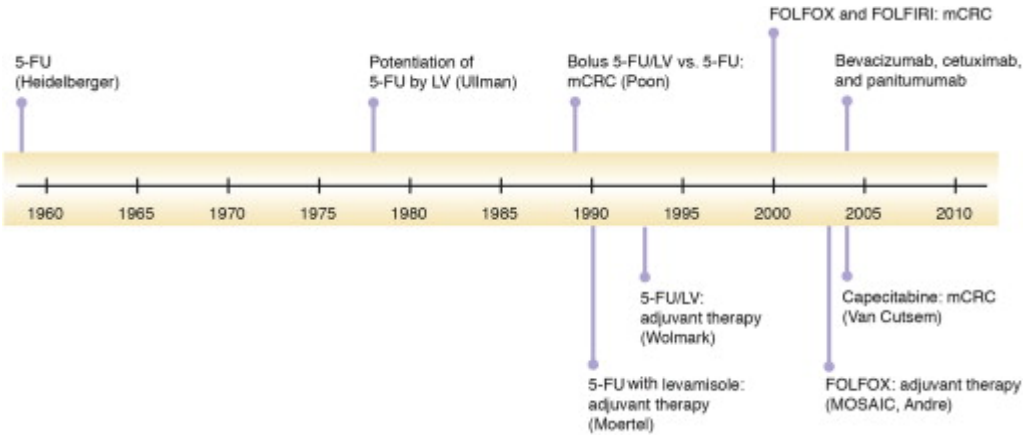
Figure 1.7 Formula for calculating NAR score taken from [157]. Pathological nodal stage (pN); Pathological tumour stage (pT); Clinical nodal stage (cN); Clinical tumour stage (cT)

$$NAR = \frac{[5\,pN - 3(cT - pT) + 12]^2}{9.61}$$

1.17 Adjuvant chemotherapy

Adjuvant systemic chemotherapy improves survival in resected stage III and some stage II cancers with negative prognostic markers such as higher risk T4, poorly differentiated tumours. Landmark studies (details in Figure 1.8) including the MOSAIC study have established oxaliplatin in addition to fluoropyrimidine (fluorouracil or capecitabine) as standard of care. [158] [159] [160] [161] The QUASAR study demonstrated a mild (3.6%) survival improvement with adjuvant with fluorouracil and folinic acid for patients with Stage 2 disease. [162] The SCOT trial demonstrated 3 months of adjuvant therapy was non-inferior to 6 months for disease free survival and had reduced treatment related toxicity in higher risk stage 2 and stage 3 patients. [163] The IDEA collaboration of the pooled analysis of 6 randomised controlled trials found only a 0.4% difference in 5 year survival between 3 and 6 months of adjuvant chemotherapy, therefore this small gain has to be balanced against significant treatment related toxicities higher in the 6 month group. [164] A large metanalysis of patients with rectal cancers from 0-15cm from the anal verge undergoing neoadjuvant chemoradiotherapy followed by complete resection did not demonstrate improved overall survival, disease free survival or distant recurrences for post-operative / adjuvant chemotherapy. Subgroup analysis of patient with cancers 10-15cm from the anal verge had improved disease free survival and fewer case of distant recurrences. [165]

Figure 1.8 showing landmark advances in colorectal cancer chemotherapy , taken from Gustaavson et al [166]. 5-FU = 5-Fluorouracil; FOLFIRI = Infusional 5-FU/LV With Irinotecan; FOLFOX = 5-FU/LV With Oxaliplatin; LV = Leucovorin; mCRC = Metastatic Colorectal Cancer; MOSAIC = Multicentre International Study of Oxaliplatin/5-FU/Leucovorin in the Adjuvant Treatment of Colon Cancer.



1.18 Systemic Inflammatory Response

Tumour and host interaction is an important determinant of the progression of malignancy. There is local inflammation within the tumour microenvironment as well as a systemic response to the tumour. These responses are controlled by a complex interaction of cytokines, chemokines, growth factors and matrix remodelling enzymes. [167, 168] Systemic inflammation is an established marker of poorer survival across multiple tumour types independent of tumour location, stage and treatment. [169]

In colorectal cancer the modified Glasgow Prognostic Score (mGPS), a scoring measure for systemic inflammation, has shown to have prognostic value independent of tumour stage, tumour pathology and comorbidities. It has also shown poorer survival and response in patients undergoing chemo/radiotherapy in colorectal and gastroesophageal cancer. The Glasgow Prognostic score takes into account C Reactive Protein (CRP), a sensitive marker of systemic inflammation and albumin. Albumin was noted to fall with increased CRP concentrations and thus a marker of systemic inflammation and lean tissue. The modified Glasgow Prognostic Score reflects Hypoalbuminaemia without elevated CRP was not common and isolated Hypoalbuminaemia did not contribute to poorer survival. [170]

Elevated mGPS has been associated with increased circulating total white cell counts, increased neutrophil counts and decreased lymphocyte counts. It was not associated with peritumoral infiltrate, which is associated with survival. [171]

Hypoalbuminaemia, elevated CRP, elevated NLR has been associated with poorer survival in operable and advanced inoperable cancer [172] [172]

Systemic inflammation is also associated with changes to white cell count causing neutrophilia and lymphopenia. A combination of these in the form of neutrophil-lymphocyte ratio (NLR) has prognostic values in a variety of cancers. An elevated NLR is

a predictor or poorer overall survival in operative and non-operative patients in a variety of cancers.[170, 173]

NLR is believed to the reflect the balance between the pro-tumour and anti-tumour activities of immune cells. Neutrophils can induce a pro-tumour effect on the local micro-environment, secreting cytokines, recruiting inflammatory cells promoting angiogenesis, invasion, tumour growth and supressing the adaptive immune response by inhibiting natural killer cells and activated T cells. Correlation between G-CSF immunoreactivity and the neutrophil count is associated with poorer survival possibly due to increased myeloid-derived suppressor cells. Cancer cells expressing g-CSF are less sensitive to radiation therapy, a phenomenon not clearly understood.[174-178]

Lymphocytes are believed to have anti-tumour effect from improved survival in rectal cancer patients who have a tumour CD4+ and CD8+ cell infiltration. Patients with high lymphocyte counts have reduced rates of local and systemic recurrence. NLR being elevated could be indicative of predominantly pro-tumour neutrophil effect or from a predominantly weaker anti-tumour lymphocyte effect.

Pre-treatment NLR <5 has been shown to have improved overall and recurrence free survival in colorectal cancer in a metanalysis by Tsai and colleagues. They also found CEA <5 was associated with improved rates of complete response to neoadjuvant chemoradiotherapy. [179] Watt and colleagues found a Neutrophil count of <7.5 was significantly associated with improved cancer specific and overall survival, however the same was not observed with NLR. [173]

Systemic inflammation in patients with cancer is mediated by circulating proinflammatory cytokines and acute phase proteins. In colorectal cancer these include increased serum IL-6, IL7, CXCL8 (IL8), PDGFB levels and reduced serum CCL2 levels. The mechanism behind this inflammatory response is complex, not clearly understood and from the

interaction of neoplastic cells and the tumour microenvironment involving inflammatory cells, fibroblasts, extracellular matrix and vasculature. [167]

Colorectal cancers are infiltrated by a various immune cell groups which make up the Tumour Microenvironment (TME). These include:

- Proinflammatory cells: CD8+ Cytotoxic T cells; type 1 $CD4^+$ helper T cells (Th1 cells); NK cells; M1 macrophages
- Anti-inflammatory cells: regulatory T cells (Treg); type 2 helper T cells (Th2 cells); M2 macrophages; myeloid derived suppressor cells (MDSCs).
- Combined pro- and anti-inflammatory effects: B lymphocytes; plasma cells; neutrophils; eosinophils; mast cells

Immune cell infiltration of the tumour microenvironment as seen on haematoxylin and eosin staining and antibody derived immunohistochemistry is widely accepted with improved survival in CRC independent of tumour stage. [167]

The link between local and systemic inflammation is not clearly understood. The local (Klintrup) and systemic (mGPS) inflammatory responses have been shown to be independently associated with cancer specific survival in the same cohort. [171]

Despite immune cells previously described being associated cytokine, chemokine and growth factor release, further studies have failed to show this, as well as inverse correlations. [167] High intra-group correlations have been shown between tumour infiltrating immune cells and cytokines but with weak inter-group correlations.

The role of systemic inflammation and radiation response is not well defined. Previous studies have demonstrated a lower post CRT leucocyte count, and greater reduction in the leucocytes following CRT in down-staged patients, however this did not reach significance for specific tumour regression grades. Further investigation into subpopulations did not

reveal any associations for neutrophil or lymphocyte counts. A lower post-CRT leukocyte count was associated with improved disease free and overall survival. [180] Ishikawa et al showed a rise in NLR from baseline to post-CRT with patients with the lowest baseline NLR having the highest post CRT NLR. Patients with high NLR post CRT had pooper pathological response than low NLR. [181] Braun et al found a high baseline NLR (>4.06) was associated with poorer DFS on multivariate analysis. [182] Zhang et al found patients with high NLR and low CD8 + T cell count had the poorest survival whilst the opposite (low NLR and high CD8+ T cell count) had the longest survival indicating a link between the local and systemic inflammatory responses. [183] Shen et al found baseline NLR>2.8 associated with poorer overall survival but not tumour response. [184]

Lymphopenia is commonly seen following radiotherapy in a variety of solid tumour types and is associated with poorer progression free survival and disease free survival . [185]. 35% of patients with rectal cancer undergoing CRT have grade III / IV lymphopenia at 2 months with lymphopenia persisting up to 12 months from treatment. [186] The total white cell counts, and its subtypes fell during treatment and recovered afterwards. Higher peripheral lymphocyte counts during neoadjuvant therapy have been associated with improved treatment response hypothesising this could be a maker of the maintenance of the host immunity during chemoradiotherapy. [187] Despite improved tumour response from tumour infiltrating T cells, this has not shown to be directly correlate with circulating lymphocyte counts.

60% of patients with colorectal cancer are iron deficient which can result in iron deficiency anaemia (IDA). IDA is likely to result from chronic GI blood loss depleting iron stores and impaired iron homeostasis from chronic inflammation. [188] [189]Iron deficiency can

impair haematopoiesis which in turn effects production of erythrocytes, immune cells such as T cells, macrophages, dendritic cells and natural killer cells (NKC) which in turn can impair the development and function of the immune system. [189] [190]Iron has in integral role in cell mediated immune response and cytokine activity which if impaired contributes to tumour immune cell evasion. [191] This can be from impaired dendritic cell function reducing activation of anti-tumour T cell response. [192] IDA also reduces the numbers of circulating T cells and impairs their motility by the inhibition of protein kinase C. IDA also result in oxidate stress which results in lymphocyte DNA damage causing defective T cell populations impairing immunosurveillance. [193, 194]IDA can cause hypoxia which downregulates NKC activation and NKA-derived granzyme B required for cancer cell elimination. [195] IDA also results in reduced circulating levels of interleukin 2 (IL2) which is critical for T cell differentiation and communication between T cells and NKCs. [196] Interferon γ (INF γ) release is impaired in IDA reducing Helper T cell activation of NKCs. Lower circulating levels of INF γ are associated with poorer survival from CRC. [197] IDA has the potential for polarising tumour associated macrophages (TAMs) decreasing M1 macrophage activity (pro-inflammatory and promote tumour regression) and increasing M1 macrophage activity (promotes tumour growth via upregulation of haemoxygenase-1 mediated iron generation and increased iron export to the tumour microenvironment.[198]) [199] In vivo models of injecting iron loaded TAMs have been able to repolarise TAMs to exert an antitumour effect. [200] [201]Iron deficiency impairs by means of iron chelation can have immunosuppressive effects via impaired activation and proliferation of regulatory T cell function within the TME resulting in uncontrolled chronic inflammation. [202]

1.19 Summary and Aims

To summarise neoadjuvant chemoradiotherapy (CRT) is widely accepted as a standard of care in managing locally advanced or margin-threatening rectal cancer, based on radiotherapy being known to downstage rectal tumours enhancing the chance of surgical cure, reducing circumferential resection margin positivity and consequently improving long term local disease control [203-206]. Despite the widespread application of neoadjuvant chemoradiation, results in terms of treatment response remain variable. Only a minority (10-20%) of patients will experience a complete pathological response and the majority of patients experience a partial or even poor response. The increased use of total neoadjuvant therapy has led to greater numbers of patients achieving a complete response allowing for organ preservation strategies.

It is important to identify responders and non-responders to allow better allocation of treatment and avoid excess toxicity where the treatment may be less effective. To date, there are no robust or reliable clinical biomarkers of treatment response with treatment allocation determined by clinical staging criteria on MRI. Various components of an elevated systemic inflammatory response in addition to anaemia and lymphopenia have previously been associated with poorer cancer outcomes and in some reports, a poorer response to neoadjuvant chemoradiotherapy. Further work is required to determine the influence of systemic inflammation on treatment response as well as whether changes in inflammatory parameters during and after treatment relate to response. This research is necessary to help in the search for biomarkers, but also to better understand how radiation response evolves in relation to systemic immune and inflammatory parameters. Novel insights into response evolution may aid the development of new strategies to augment or improve radiotherapy response.

The aims of this thesis are to:

1) Better understand the relationship between pre and post systemic inflammatory parameters and neoadjuvant chemoradiation treatment response in rectal cancer. (Chapter 2)
2) Improve our understanding of how changes in systemic inflammation during and after treatment impact treatment response to neoadjuvant chemoradiation in rectal cancer (Chapter 2)
3) Understand the role of pre-treatment lymphopenia on response to neoadjuvant chemoradiation in rectal cancer (Chapter 3)
4) Understand the role of pre-treatment anaemia on response to neoadjuvant chemoradiation in rectal cancer
5) Design and implement a prospective study which incorporates serial sampling during neoadjuvant therapy in rectal cancer to chart temporal changes in local and systemic immune/ inflammatory profiles and these features impact on treatment response.

2 An investigation into the influence of the circulating markers of systemic inflammatory response on response to neoadjuvant chemoradiotherapy

2.1 Introduction

Neoadjuvant chemoradiotherapy (CRT) is widely accepted as a standard of care in managing locally advanced or margin-threatening rectal cancer. The systemic inflammatory repones (SIR) is a validated, stage independent predictor of long term survival in in gastrointestinal cancers (Section 1.20 in Chapter 1 Introduction). Results from previous modest sized cohort studies examining circulating C-Reactive protein (CRP) and, the neutrophil lymphocyte ratio (NLR), have reported variable results, with inconsistent definitions of normal values, inclusion criteria and staging. [169, 173, 207-209].

When measured at baseline (prior to commencing neoadjuvant therapy), Carruthers et al has previously reported NLR <5 is associated with improved disease free and overall survival but did not report on associations between NLR and tumour response. [210] Kim et al reported an elevated baseline (pre-treatment) NLR (>3), larger tumour size (3cm) and higher CEA (>5) were associated with poorer neoadjuvant therapy response (ypTNM>1). These factors were also associated with improved disease free and overall survival.

Other reports in the pre-operative setting (after neoadjuvant therapy) suggest an elevated modified Glasgow Prognostic Score (mGPS) and NLR >5 has been associated with a poorer pathological response. [211] [212]. Other circulating parameters including CEA, platelet count, lymphocyte count, PLR and NLR are have also been reported to relate to treatment response [213] [214]. Krauthamer et al reported Albumin >35 and NLR <5 was

associated with pCR in patients with clinical stage III disease but not in stage II or all patients. [215] Elevated levels of systemic inflammatory parameters including NLR and PLR are associated with more advanced disease staging and therefore it is not clear whether these circulating parameters reflect disease stage or whether there is a biological link to radioresistance.

In recent years, response to neoadjuvant therapy has been measured using pathological tumour response, and neoadjuvant rectal (NAR) score discussed in 1.16.4 of the introduction. Good or complete pathological response has been shown to be a good prognostic marker. [216, 217] The relationships between measures of the systemic inflammatory response measured prior to and at the end of treatment with the NAR score has not previously been determined. It would be important to perform such an assessment in a large cohort of patients staged uniformly with MRI and who were treated with a consistent neoadjuvant regimen.

The aim of this chapter is to comprehensively assess the relationship between biomarkers of systemic inflammation at three time points (baseline, immediately following administration of neoadjuvant therapy and preoperatively) and response to neoadjuvant therapy. Specifically, the inter-relationships between clinical staging, the systemic inflammatory response and treatment response using modern assessment criteria including NAR will be assessed. The literature thus far has mainly concentrated on markers of SIR at specific time points. In addition, the relationship between temporal changes in markers of systemic inflammation and how these relate to clinicopathological factors and treatment response to neoadjuvant chemoradiotherapy was investigated. We hypothesis that systemic inflammation and increasing inflammation during neoadjuvant therapy will be associated with poorer response to nCRT.

2.2 Methods

Two prospectively collected databases were combined and used to identify the population of interest for the present study. First, a prospectively collected and maintained database of all patients with colorectal cancer treated at Glasgow Royal Infirmary from 2008 and 2014 and secondly, a database of all patients who received radiotherapy for rectal cancer at the West of Scotland Beatson Cancer Centre between 2014-2016. The latter was a list of Community Health Index numbers. For the creation of this combined dataset, I collected blood results and comprehensive clinico-pathological data for these patients from electronic patient records. Patients receiving neoadjuvant long course chemoradiotherapy (CRT) for non-metastatic disease followed by potentially curative resection for rectal cancer from were identified from these databases. Caldicott guardian and West of Scotland Research Ethics Committee approval was sought to enable data collection on clinical and pathological staging and treatment received for the present study.

During this period, common indications for neoadjuvant CRT were clinical circumferential resection margin threat on MRI, nodal disease or low rectal cancers which would result in an abdominoperineal resection. All patients were staged using pre-treatment computed tomography and treatment decisions made at a multidisciplinary team meeting. Specifically, patients with non-metastatic rectal cancer, with fitness and disease characteristics deemed appropriate for a curative treatment plan were selected. Patients who were unsuitable for a curative resection, those with metastatic disease, unsuitable for long course CRT, had previous surgery for rectal cancer, a complete clinical response following neoadjuvant therapy or chronic inflammatory conditions (such as inflammatory bowel disease or chronic polyarthritis) were excluded.

All patients had biopsy proven rectal adenocarcinoma underwent pre-treatment staging with CT and rectal MRI. At completion of neoadjuvant therapy, all patients underwent repeat CT staging and most (70%) of patients underwent repeat rectal MRI. Clinical

tumour characteristics were based on pre-treatment MRI. Radiological response and downstaging on post-CRT MRI compared to pre-CRT MRI was determined by colorectal radiologists based on MRI tumour regression grade (mrTRG) detailed in section 1.18.2 of the introduction, mrTRG grade 1 or 2 was deemed a good response, mrTRG grade 3 was deemed an incomplete response and mrTRG grade 5 or 6 was deemed a poor response.

The neoadjuvant treatment regimen was decided by the treating clinical oncologist. Radiotherapy dose was usually 45Gy in 25 fractions over 5 weeks with 3 patients receiving a boost to regional nodes (range 32-54Gy). Concomitant chemotherapy was oral capecitabine, intravenous 5-Flurourcail or capecitabine. Combining this with irinotecan or oxaliplatin was undertaken in 3 study patients, detailed later.

Measures of systemic inflammatory parameters were obtained from routine standard of care blood tests. Blood tests taken in the 1-3 weeks before commencing CRT were defined as baseline bloods. Blood tests taken from the last week of radiotherapy to 4 weeks after completing CRT were defined as post-treatment bloods. Blood tests taken 12-20 weeks after commencing CRT and within 2 weeks of surgery were defined as pre-operative bloods. Blood investigations included full blood count incorporating a differential white cell count, routine biochemistry, C-reactive protein and carcinoembryonic antigen. All lab results were grouped according to standard thresholds. Changes in values were calculated from baseline to following CRT and from baseline to pre-op. Static was defined as an increase or decrease of less than 1 standard deviation (sd). Decrease was defined as a decrease of greater than 1 sd. Increase was defined as an increase of greater than 1 sd. Neutrophil to Lymphocyte ratio was obtained from the differential blood count by dividing the circulating neutrophil count by circulating lymphocyte count. Modified Glasgow prognostic score (mGPS) was constructed using methods previously described within the introduction section 1.20. [218]

To further study broader changes in NLR, CRP, CEA and mGPS, they were dichotomised into low (normal) and high (elevated) as per widely accepted cutoffs. In an attempt to study trends between time points 2 further groups were created:

1) No inflammation as baseline: Those at baseline who were not inflamed and developed inflammation, which includes serum markers remaining low or increasing from low to high (Low Low or Low High) between time points.
2) Inflammation at baseline: Those at baseline who were but became less / non-inflamed, which includes serum markers remain high or decreasing from high to low (High High or High Low) between time points.

Electronic operative records were reviewed, and details of operative approach and post operative outcomes were collected. Patients who underwent a laparoscopic to open procedure were defined as laparoscopic assisted.

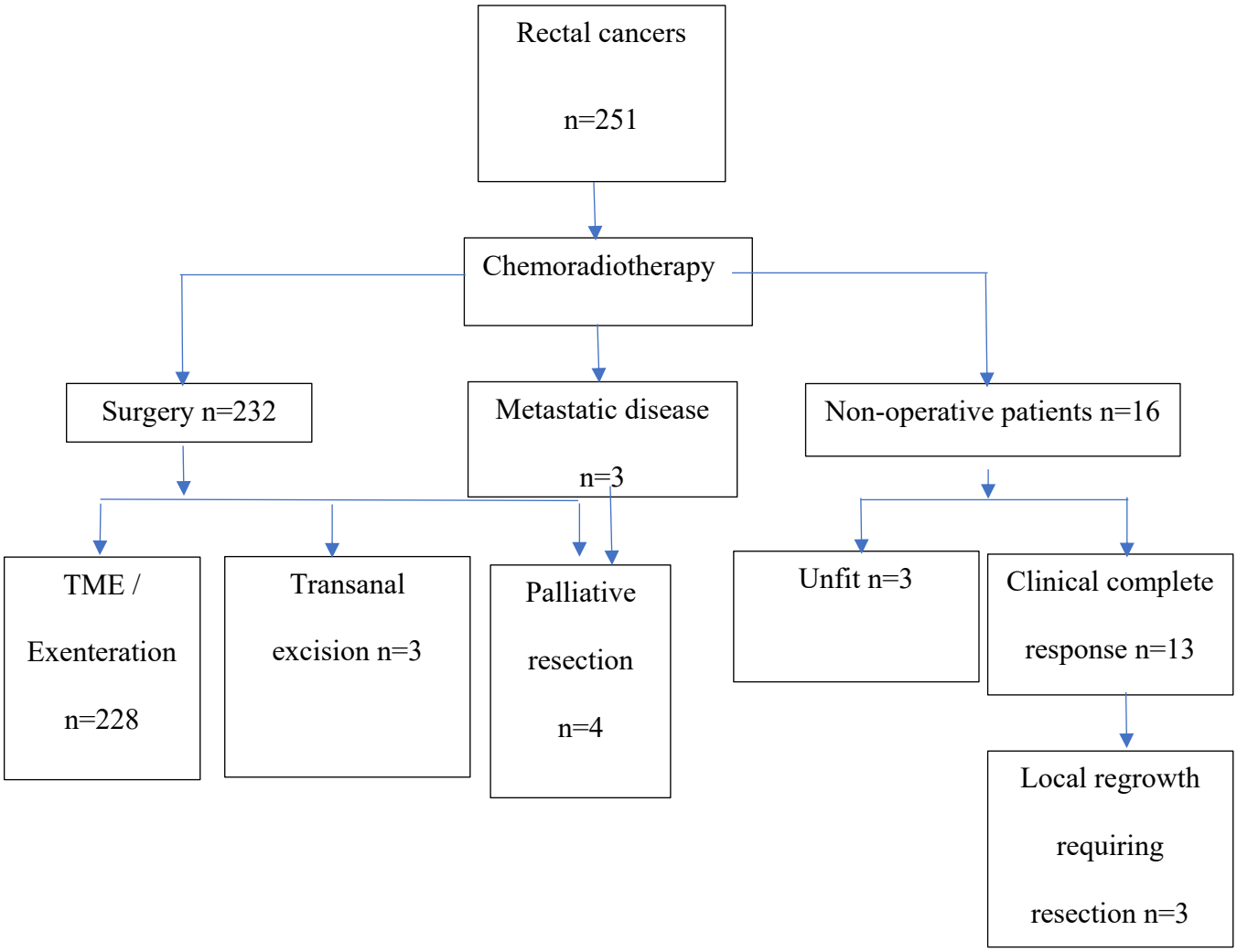
Pathological tumour response from chemoradiotherapy was defined as per the Royal College of Pathologist colorectal cancer dataset based on the 4 tier system described by Ryan et al. [216, 217] (Chapter 1 Section 1.18.1). Score of 0 if there were no viable cancer cells (complete response). Score 1 if there were single cells or small groups of cancer cells (near-complete response). Score 2 if residual cancer with evident tumour regression but more than single cells or small groups of cancer cells (partial response). TRG 3 if there is extensive residual cancer with no evidence of tumour regression (poor response). [217] Tumour response was dichotomised into good / near complete response (TRG 0/1) and incomplete / poor response (TRG 2/3). Neoadjuvant rectal (NAR) score was calculated using clinicopathological parameters (detailed in Chapter 1 section 1.18.4), stratifying patients into low (<8), intermediate (8-16) and high (>16) risk groups. [157] MRI T or N downstaging was defined as a reduction in T or N stage on post treatment imaging compared to pre-treatment assessment.

Statistical analysis of the whole dataset was undertaken by me using SPSS (version 25.0; IBM SPSS Statistics, IBM corp, Armonk, NY, USA). Variables were grouped using standard clinical thresholds or cut offs which have been established in literature. Comparison between groups was performed with the Chi square test, and Fisher's exact test was used where n was less than 5. Kruskal-Walli's assessment of variance was used for the comparison of median values. Freidman test was used for assessment of variance in mean values from 3 variables. Binary logistic regression was used for univariate and multivariate analysis of baseline characteristics and their relationship with treatment response (tumour regression grade / NAR score / pathological complete response). In order to gain entry to the multivariate model, a threshold of $P<0.05$ on univariate analysis was required.

2.3 Results

This study identified a total of 251 patients who had undergone long course CRT of whom 235 were managed surgically and 16 were initially managed non-operatively. A flow chart detailing the treatment course and outcome of patients included is shown in Figure 1. Of patients undergoing primary non-operative treatment, 3 were not suitable for surgery due to co-morbidity and 13 had a complete clinical response (cCR). Of the 13 cCRs, 3 patients developed local regrowth and underwent salvage resection. Of the patients managed surgically, four had a palliative resection and 3 had a transanal excision. The remaining 228 having a resection with curative intent were studied in detail within this and subsequent chapters.

Figure 2.1. Patient selection for this the study.



A total of 251 patients were identified. Two hundred and thirty two patients underwent surgery of which 228 patients underwent a resection with curative intent, 3 underwent transanal excision with curative intent and 1 patient had a palliative resection. Three patients did not develop metastatic disease and went on to have resection. Sixteen patients were initially treated non-operatively, 3 of whom were did not undergo intensive surveillance due to comorbidity. Thirteen patents had a complete clinical response (cCR), of whom 3 had tumour regrowth and underwent a salvage resection. Ten remaining patients had a sustained cCR requiring no further intervention.

Table 2.1 Baseline clinical and pathological details for 228 patients who received neoadjuvant chemoradiotherapy rectal cancer in the West of Scotland from February 2008 to May 2016. Numbers in parentheses indicate percentages.

		Frequency (%)
Age (years)	<55	44 (19)
	55 – 74	144 (63)
	≥75	40 (18)
Sex	Female	88 (39)
	Male	140 (61)
BMI	<25	70 (39)
	25-30	63 (35)
	>30	46 (26)
Tumour Height from anal verge (cm)	<5 (low)	93 (43)
	5 – 10 (mid)	81 (37)
	>10 (upper)	43 (20)
Tumour Size (cm)	<4	35 (16)
	4-7	163 (74)
	≥8	23 (10)
Clinical T Stage	T2	15 (7)
	T3	173 (76)
	T4	40 (18)
Clinical N Stage	N0	66 (28)
	N1/2	164 (72)
TNM stage	I	5 (2)
	II	59 (26)
	III	164 (72)
MRI EMVI n=103	No	39 (38)
	Yes	64 (62)
MRI Response n=146	Poor (mrTRG 4 & 5)	11 (7)
	Incomplete (mrTRG 3)	68 (47)
	Good (mrTRG 1&2)	67 (46)

MRI T Downstaging n=77	No	34 (44)
	Yes	43 (56)
MRI N Downstaging n=77	No	29 (38)
	Yes	48 (62)
RT Dose (Gy)	<45	8 (4)
	45	189 (95)
	>45 (boost)	3 (1)
Concomitant Chemotherapy	Capecitabine	150 (87)
	5-FU	19 (11)
	Other *	3 (1)
Interval: CRT finish to Surgery (weeks)	≤8	24 (11)
	8-12	128 (56)
	>12	75 (33)
Operation	TME with primary anastomosis	86 (38)
	TME with end colostomy	20 (9)
	Abdominoperineal resection (APR)	117 (52)
	Pelvic Exenteration **	5 (2)
Operative approach	Laparoscopic	45 (20)
	Laparoscopic assisted	22 (10)
	Open	161 (70)
Permanent Stoma	No	75 (33)
	Yes ***	153 (67)
Pathological T staging	ypT0	45 (20)
	ypT1	12 (5)
	ypT2	40 (17)
	ypT3	116 (51)
	ypT4	15 (7)
Pathological N staging	ypN0	163 (72)
	ypN1	49 (21)
	ypN2	16 (7)
pCR	No	183 (80)
	Yes	45 (20)

NAR score	<8 (low risk)	52 (23)
	8-16 (intermediate risk)	108 (47)
	>16 (high risk)	68 (30)
Tumour Regression Grade	TRG 0 (pCR)	45 (21)
	TRG 1	35 (16)
	TRG 2	85 (39)
	TRG 3	52 (24)
Resection Margin	R0	201 (88)
	R1 (≤1mm to CRM)	27 (12)
EMVI	No	160 (74)
	Yes	56 (26)
Tumour differentiation	Well, / Moderate	190 (72)
	Poor	17 (8)
Adjuvant chemotherapy	No	158 (70)
	Yes	68 (30)

BMI body mass index; TNM American Joint Committee Cancer (AJCC) TNM system; EMVI extramural vascular invasion; RT radiotherapy; CRT chemoradiotherapy; 5-FU Fluorouracil; pCR pathological complete response; NAR Neoadjuvant rectal score; TRG AJCC tumour regression score quantifies tumour regression to neoadjuvant chemoradiotherapy *concomitant chemotherapy other: 1 Capecitabine & Irinotecan; 2 Xelox (Oxaliplatin & Capecitabine)

** Pelvic exenteration: 3 APR & cytoprostatectomy; 2 APR & cystectomy & TAH & BSO

***Includes 7 patients who did not have a reversal of diversion ileostomy

The majority of patients were over 55 years of age (n=154, 81%), male (n=140, 61%) and had a BMI of less than 30 (n=133, 74%). The majority of tumours were in the low or mid rectum (n=174, 80%) and between 4-7cm in size (n=163, 74%). The majority had clinical T3 disease (n=173, 76%), and (n=164 72%) had Stage III disease. Of the 5 stage 1 patients, 4 had low rectal cancers deemed to require an APR whilst 1 had a mid-rectal cancer. Extramural venous invasion (EMVI) on MRI was reported in 106 patients of whom the majority had EMVI (64, 62%). MRI response was classified a poor (mTRG 4&5), incomplete (mTRG3), good (mTRG 1&2) (n=11 7%, n=68 47%, n=67 46%). [219] The majority of patients received Capecitabine or in combination with irinotecan or oxaliplatin (n=153, 88%). Nearly all patients (n=192, 96%) received a total radiation dose of 45Gy or more. Most patients (n=203, 89%) had an interval of over 8 weeks between finishing radiotherapy and surgery.

Fifty two percent (n=117) of the cohort underwent a sphincter-excising abdominoperineal resection (APR)). Fort six percent (n=105) of the cohort underwent a sphincter preserving TME resection, the majority of whom (n=87, 83%) had a primary anastomosis. Five patients had a pelvic exenteration. The majority of patients underwent open surgery (n=161, 70%) and 153 patients (67%) had a permanent stoma which included 7 patients who did not have reversal of defunctioing ileostomy. The majority of patients had a pathological staging of ypT3 (n=116, 51%) and were node negative (n=163, 72%). Forty-five patients (20%) had a complete pathological response (pCR). Near complete response (TRG 1) was seen in 35 patients (16%) and a poor response (TRG 3) in 52 patients (24%). Twenty seven patients (12%) had margin involvement (R1), 56 (24%) had extra-mural vascular invasion and 18 (9%) had poor tumour differentiation. The majority of patients (n=160, 70%) had low or intermediate NAR scores. Sixty-eight patients (30%) received adjuvant chemotherapy. Detailed baseline clinical and pathological information is detailed in table 2.1. The baseline characteristics of the 2 cohorts used for the main datasets are

detailed in table 2.1s located within the appendix. A subgroup analysis of baseline clinical and pathological characteristics of both cohorts which constitute the main dataset reveal the larger West of Scotland cohort had more patients with a BMI>30, clinically node positive disease, clinical TNM stage III disease, patients waiting >12 weeks for surgery after the finishing neoadjuvant therapy and a trend towards lower number of pathological N2 than the Glasgow Royal Infirmary cohort. No differences were seen in age, gender, tumour height, tumour size, clinical T stage, radiation dose, type of chemotherapy, type of surgery, pathological TNM stating, margin involvement and pathological tumour regression grading. The full table is within the appendix. The median time between pre-CRT and post CRT blood tests was 5 weeks. The median time between post-CRT and pre-operative blood tests was 11 weeks. The median time between pre-CRT and pre-operative blood tests was 17 weeks.

Table 2.2 details the changes in circulating measures of the systemic inflammatory response in addition to other parameters (e.g. CEA) measured at different time points during the neoadjuvant treatment pathway, Across the whole cohort, during CRT white cell count, neutrophil count and lymphocyte counts decreased and recovered slightly (detailed in table 3a). A similar trend was observed in haemoglobin and albumin. NLR increased during radiotherapy, but the rate of increase decreased between the completion of radiotherapy and surgery. CEA decreased during CRT. Proportion of patients with a CRP >10 increased during CRT. This was reflected in the proportion of patients who had an elevated mGPS during therapy. Changes in blood parameters of patients with blood samples at all 3 time points was available in 144 patients is detailed in table 2.2.

Table 2.2 Circulating measures of the systemic inflammatory response prior to and following neoadjuvant therapy in matched patients (with blood results available at all 3 time points) with rectal cancer (n=144). Median and IQR in parenthesis for continuous data. Numbers in parentheses indicate percentages.

		Baseline (pre-treatment bloods)	Post Radiotherapy (last week of RT to 4 weeks post RT)	Pre-operative (8-16 weeks post RT)	P value
WCC (10^9L)		7.6 (3.0)	5.3 (2.8)	5.6 (2.1)	<0.001 ◊
n=144	<4 / 4-11 / >11	4 (3) / 129 (88) / 13 (9)	30 (21) / 113 (78) / 2 (1)	19 (13) / 124 (86) / 2 (1)	0.028 #
Neutrophils (10^9L)		4.7 (2.0)	3.4 (2.1)	3.8 (2.2)	<0.001 ◊
n=144	<2 / 2-7.5 >7.5	1 (1) / 123 (84) / 22 (15)	13 (9) / 126 (87) / 6 (4)	3 (2) / 143 (92) / 9 (6)	0.016 #
Lymphocytes (10^9L)		1.9 (1.1)	0.7 (0.5)	0.9 (0.5)	<0.001 ◊
n=144	<1.5 / 1.5-4 / >4	43 (30) / 98 (67) / 5 (3)	137 (95) / 7 (5) / 1 (1)	127 (87) / 18 (12) / 1 (1)	0.003 #
NLR		2.7 (1.8)	4.9 (3.0)	5.2 (3.0)	<0.001 ◊
n=144	<3 / ≥3	87 (60) / 59 (40)	22 (15) / 123 (85)	47 (32) / 99 (68)	0.001 #
Hb (g/dL)		135 (21)	129 (20)	130 (19)	<0.001 ◊
Anaemia Hb <130g/dL (men) <115g/dL (women) n=144	No/Yes	118 (81) / 28 (19)	97 (67) / 48 (33)	111 (77) / 34 (23)	<0.001 #
Albumin (g/L)		38 (5)	36 (4)	37 (6)	<0.001 ◊

n=141	<35 / ≥35	26 (18) / 120 (82)	43 (30) / 101 (70)	30 (21) / 113 (79)	<0.001 #
CEA (µg/L)		3.7 (8.7)	2.9 (5.8)	NA	<0.001 √
n=99	<5 / ≥5	60 (61) / 39 (39)	73 (70) / 32 (31)	NA	<0.001 #
CRP (mg/L)		3.4 (5.0)	3.0 (5.0)	4.0 (4)	0.181 ◊
n=80	≤10 / >10	68 (85) / 12 (15)	93 (80) / 24 (20)	70 (83) / 14 (17)	0.021 #
mGPS n=80	0 / 1/ 2	68 (85) / 5 (6) / 7 (9)	93 (79) / 8 (7) / 16 (14)	70 (83) / 5 (6) / 9 (11)	0.001 #

RT radiotherapy; WCC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein; mGPS modified Glasgow prognostic score. ◊ Freidman test. √ Kruskal-Wallis test. # Chi-Squared test

Tumour Regression Grade

The relationships between circulating measures of the systemic inflammatory response and tumour regression grading are detailed in table 2.3.1. A higher pre-operative neutrophil count trended (p=0.056) towards an improved tumour regression to neoadjuvant treatment. This was not observed with the neutrophil count at other time points. A lower haemoglobin and anaemia were associated with a poorer tumour regression at all 3 time points. Improved tumour regression was associated with a larger proportion of patients with normal pre-operative serum albumin. This was not observed with serum albumin at other time points. A low CEA related to a good response at both time points. WCC, lymphocyte count, NLR, CRP, mGPS were not associated with tumour regression grade at any time point.

Table 2.3.1 The relationship between circulating measures of the systemic inflammatory response measures prior to and following treatment and response to neoadjuvant therapy evaluated by tumour regression grade. Median and IQR in parenthesis for continuous data. Numbers in parentheses indicate percentages.

			Tumour Regression Grade		P value
			Good (TRG 0/1)	Partial / Poor (TRG 2 / 3)	
WCC (109L)	Pre-CRT		7.3 (2.6)	8.3 (3.1)	0.449
	n=222	<4 / 4-11 / >11	1 (1) / 71 (89) / 8 (10)	4 (3) / 118 (87) / 14 (10)	0.713
	Post-CRT		4.4 (3.2)	5.3 (2.3)	0.516
	n=216	<4 / 4-11 / >11	17 (22) / 58 (76) / 1 (1)	24 (18) / 108 (81) / 1 (1)	0.709
	Pre-Op		5.6 (2.9)	5.5 (1.8)	0.837
	n=230	<4 / 4-11 / >11	11 (14) / 67 (84) / 2(2)	16 (12) / 116 (87) / 1 (1)	0.544
Neutrophils (109L)	Pre-CRT		4.5 (1.5)	5.3 (2.0)	0.056
	n=222	<2 / 2-7.5 >7.5	0 / 67 (84) / 13 (16)	1 (1) / 115 (85) / 20 (15)	0.720
	Post-CRT		3.2 (2.9)	3.6 (1.8)	0.311
	n=216	<2 / 2-7.5 >7.5	7 (9) / 67 (88) / 2 (3)	11 (8) / 117 (88) / 5 (4)	0.888
	Pre-Op		3.8 (2.5)	3.8 (2.1)	0.822
	n=230	<2 / 2-7.5 >7.5	1 (1)/ 71 (89) / 8 (10)	5 (4) / 125 (93) / 4 (3)	0.063
			1.9 (1.1)	1.7 (1.3)	0.140

Lymphocytes (109L)	Pre-CRT n=222	<1.5 / 1.5-4 / >4	20 (25) / 57 (71) / 3 (4)	49 (36) / 84 (62) / 3 (2)	0.200
	Post-CRT n=216		0.6 (0.6)	0.8 (0.6)	0.709
		<1.5 / 1.5-4 / >4	70 (92) / 6 (8) / 0	127 (96) / 5 (4) / 1(1)	0.342
	Pre-Op n=230		0.8 (0.4)	1.0 (0.6)	0.403
		<1.5 / 1.5-4 / >4	70 (88) / 10 (13) / 0	119 (89) / 14 (10) / 1 (1)	0.679
NLR	Pre-CRT n=222		2.5 (2.0)	3.1 (2.3)	0.334
		<3 / ≥3	50 (62) / 31 (38)	76 (56 (40 (44)	0.477
	Post-CRT n=216		5.9 (5.6)	4.7 (3.1)	0.534
		<3 / ≥3	16 (21) / 61 (79)	19 (14) / 114 (86)	0.251
	Pre-Op n=230		4.1 (4.4)	4.1 (3.6)	0.610
		<3 / ≥3	27 (33) / 54 (67)	42 (31) / 92 (69)	0.765
Hb (g/dL) Categorical Hb Hb <130g/dL (men) <115g/dL (women)	Pre-CRT n=222		137 (22)	129 (19)	0.008
		NO / YES	73 (90) / 8 (10)	94 (69) / 42 (31)	<0.001
	Post-CRT n=216		129 (33)	125 (18)	<0.001
		NO / YES	57 (74) / 20 (26)	72 (54) / 61 (46)	0.005
	Pre-Op n=230		136 (25)	131 (17)	0.018
		NO / YES	70 (86) / 11 (14)	93 (70) / 40 (30)	0.008
			37 (6)	37 (6)	0.513

Albumin (g/L)	Pre-CRT n=222	<35 / ≥35	16 (20) / 64 (80)	29 (21) / 107 (79)	0.864
	Post-CRT n=216		36 (4)	36 (4)	0.122
		<35 / ≥35	19 (24) / 59 (76)	46 (35) / 86 (65)	0.124
	Pre-Op n=186		37 (4)	37 (4)	0.016
		<35 / ≥35	12 (15) / 66 (85)	36 (28) / 94 (72)	0.043
CEA (µg/L)	Pre-CRT n=157		2.7 (1.7)	4.4 (10)	0.057
		<5 / ≥5	46 (74) / 16 (26)	45 (52) / 41 (48)	0.018
	Post-CRT n=186		2.7 (1.8)	3.8 (9.0)	0.003
		<5 / ≥5	48 (83)/ 10 (17)	57 (60) / 38 (40)	0.004
CRP (mg/L)	Pre-CRT n=129		3 (3)	4 (8)	0.521
		≤10 / >10	37 (90) / 4 (10)	66 (80) / 17 (20)	0.203
	Post-CRT n=186		2.1 (6)	3 (10)	0.089
		≤10 / >10	53 (82) / 12 (18)	78 (71) / 32 (29)	0.149
	Pre-Op n=143		2.4 (4)	4 (4)	0.639
		≤10 / >10	44 (88) / 6 (12)	70 (84) / 13 (16)	0.618
mGPS	Pre-CRT n=124	0 / 1 / 2	37 (90) / 2(5) / 2(5)	66 (79) / 8 (10) / 9 (11)	0.324
	Post-CRT	0 / 1 / 2	53 (81) / 4 (6)/ 8 (13)	78 (71) / 10 (9) / 22 (20)	0.291

	n=175				
	Pre-Op n=133	0 / 1 / 2	44 (88) / 3 (6) / 3 (6)	70 (84) / 6 (7) / 7 (8)	0.833

TRG AJCC tumour regression score quantifies tumour regression to neoadjuvant chemoradiotherapy; WCC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein; mGPS modified Glasgow prognostic score

NAR score The relationships between circulating measures of the systemic inflammatory response and NAR <8 is detailed in table 2.3.2. NAR <8 was associated with a higher post-treatment haemoglobin. Patients meeting the criteria for anaemia at baseline and pre-operatively were less likely to have NAR scores <8. NAR<8 was associated with a pre- and post-treatment CEA <5. WCC, Neutrophil count, Lymphocyte count, NLR, Albumin, CRP and mGPS were not associated with NAR <8.

Table 2.3.2 The relationship between circulating measures of the systemic inflammatory response measures prior to and following treatment and response to neoadjuvant therapy evaluated by Neoadjuvant Response (NAR) score. Median and IQR for continuous data. Numbers in parentheses indicate percentages

			NAR Score		P value
			<8 (good)	>8 (intermediate / poor)	
WCC (109L)	Pre-CRT n=222		7.3 (3.1)	7.6 (2.7)	0.904
		<4 / 4-11 / >11	1 (2) / 47 (90) / 4 (8)	4 (2) / 153 (87) / 19 (11)	0.764
	Post-CRT n=216		5.8 (3.5)	5.3 (2.8)	0.745
		<4 / 4-11 / >11	9 (18) / 40 (80) / 1 (2)	36 (21) / 133 (78) / 2 1(1)	0.820
	Pre-Op n=230		5.7 (1.9)	5.5 (2.4)	0.429
		<4 / 4-11 / >11	3 (6) / 48 (92) / 1 (2)	25 (14) / 146 (84) / 2 (1)	0.236
Neutrophils (109L)	Pre-CRT n=222		5.7 (1.9)	5.1 (1.7)	0.393
		<2 / 2-7.5 >7.5	0 / 44 (85) / 8 (15)	1 (1) / 148 (84) / 27 (15)	0.863
	Post-CRT n=216		4.2 (1.4)	3.5 (1.8)	0.975
		<2 / 2-7.5 >7.5	4 (8) / 44 (88) / 2 (4)	15 (9) / 150 (88) / 6 (3)	0.974
	Pre-Op n=230		3.9 (2.9)	4.0 (2.3)	0.611
		<2 / 2-7.5 >7.5	0 / 48 (92) / 4(8)	6 (3) / 158 (91) / 10 (6)	0.359

Lymphocytes (109L)	Pre-CRT n=222		2.0 (1.4)	1.7 (0.9)	0.139
		<1.5 / 1.5-4 / >4	12 (23) / 37 (71) / 3(6)	60 (34) / 113 (75) / 3 (2)	0.113
	Post-CRT n=216		0.6 (0.7)	0.8 (0.6)	0.329
		<1.5 / 1.5-4 / >4	45 (90) / 5 (10) / 0	164 (96) / 6 (3) / 1 (1)	0.156
	Pre-Op n=230		1.1 (0.8)	0.8 (0.5)	0.054
		<1.5 / 1.5-4 / >4	44 (85) / 8 (15) / 0	142 (83) / 28 (16) / 1 (1)	0.270
NLR	Pre-CRT n=222		2.8 (2.7)	2.9 (2.2)	0.211
		<3 / ≥3	31 (60) / 21 (40)	102 (58) / 74 (42)	0.831
	Post-CRT n=216		6.5 (8.3)	4.8 (2.9)	0.476
		<3 / ≥3	11 (22) / 39 (78)	25 (15) / 146 (85)	0.214
	Pre-Op n=230		3.6 (3.3)	4.1 (3.7)	0.460
		<3 / ≥3	19 (36) / 33 (64)	55 (32) / 119 (68)	0.506
Hb (g/dL) Anaemia Hb <130g/dL (men) <115g/dL (women)	Pre-CRT n=222		139 (20)	129 (22)	0.027
		NO / YES	48 (92) / 4 (8)	128 (73) / 48 (27)	0.003
	Post-CRT n=216		129 (32)	125 (24)	0.039
		NO / YES	36 (72) / 14 (28)	100 (59) / 71 (41)	0.084
	Pre-Op n=230		135 (24)	132 (19)	0.052
		NO / YES	44 (85) / 8 (15)	126 (73) / 47 (27)	0.083

Albumin (g/L)	Pre-CRT n=222		38 (7)	37 (6)	0.198
		<35 / ≥35	10 (19) / 42 (81)	27 (21) / 138 (79)	0.765
	Post-CRT n=216		36 (4)	36 (4)	0.024
		<35 / ≥35	13 (26) / 37 (74)	56 (33) / 115 (67)	0.365
	Pre-Op n=186		37 (4)	37 (4)	0.012
		<35 / ≥35	8 (17) / 40 (83)	42 (25) / 129 (75)	0.250
CEA (µg/L)	Pre-CRT n=157		2.6 (2.1)	3.7 (9.1)	0.003
		<5 / ≥5	34 (79) / 9 (21)	61 (56) / 49 (45)	0.007
	Post-CRT n=186		2.0 (1.8)	3.6 (9.6)	0.005
		<5 / ≥5	32 (87) / 5 (13)	76 (62) / 46 (38)	0.006
CRP (mg/L)	Pre-CRT n=129		3.0 (1.0)	4 (7)	0.541
		≤10 / >10	24 (92) / 2 (8)	82 (81) / 19 (19)	0.173
	Post-CRT n=186		2.0 (2.0)	3 (10)	0.230
		≤10 / >10	35(83) / 7(17)	103 (72) / 40 (28)	0.139
	Pre-Op n=143		1.7 (4)	4 (4)	0.291
		≤10 / >10	27 (84) / 5(16)	92 (85) / 16 (15)	0.910
mGPS	Pre-CRT n=124	0 / 1 / 2	24 (92) / 1 (4) / 1 (4)	82 (81) / 9 (9) / 10 (10)	0.396
	Post-CRT n=175	0 / 1 / 2	35 (83) / 1 (2) / 6 (14)	103 (72) / 14 (10) / 26 (18)	0.219

	Pre-Op n=133	0 / 1 / 2	27 (84) / 3 (9) / 2 (6)	92 (85) / 8 (7) / 8 (7)	0.919

NAR Neoadjuvant rectal score; WCC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein; mGPS modified Glasgow prognostic score

Pathological Complete Response

The relationships between circulating measures of the systemic inflammatory response and pathological complete response (pCR)) are detailed in table 2.3.3. A higher proportion of patients with a pCR pathological complete response (pCR) had a normal pre-operative WCC but not at other time points. pCR was associated with absence of anaemia and higher haemoglobin, and Lower CEA. Neutrophil count, Lymphocyte count, NLR, Albumin, CRP and mGPS were not associated with pCR.

Table 2.3.3 The relationship between circulating measures of the systemic inflammatory response measures prior to and following treatment and response to neoadjuvant therapy evaluated by pathological complete response. Median and IQR for continuous data. Numbers in parentheses indicate percentages

			Pathological Complete Response		
			Yes	No	P value
WCC (109L)	Pre-CRT n=222		8.3 (2.7)	7.5 (2.8)	0.948
		<4 / 4-11 / >11	1 (2) / 40 (89) / 4 (9)	4 (2) / 160 (87) / 19 (10)	0.957
	Post-CRT n=216		5.8 (3.5)	5.3 (2.7)	0.947
		<4 / 4-11 / >11	8 (18) / 35 (80) / 1 (2)	37 (21) / 138 (78) / 2 (1)	0.787
	Pre-Op n=230		6.0 (1.8)	5.4 (2.4)	0.867
		<4 / 4-11 / >11	0 / 44 (98) /1(2)	28 (16) / 150 (83) / 2 (1)	0.017
Neutrophils (109L)	Pre-CRT n=222		4.2 (1.8)	5.0 (1.7)	0.739
		<2 / 2-7.5 >7.5	0 / 37 (82) / 8 (18)	1(1) / 155 (84) / 27 (14)	0.784
	Post-CRT n=216		3.7 (2.6)	3.5 (1.7)	0.416
		<2 / 2-7.5 >7.5	3 (7) / 39 (89) / 2 (4)	16 (9) / 155 (88) / 6 (3)	0.845
	Pre-Op n=230		4.2 (1.9)	3.8 (2.2)	0.947
		<2 / 2-7.5 >7.5	0 / 41 (91) /4 (9)	6 (3) / 165 (91) / 10 (6)	0.341
	Pre-CRT		2.2 (1.2)	1.7 (0.9)	0.388

Lymphocytes (109L)	n=222	<1.5 / 1.5-4 / >4	10 (22)/ 32 (71) / 3(7)	62 (34) / 118 (64) / 3 (2)	0.072
	Post-CRT		0.5 (1.0)	0.8 (0.6)	0.687
	n=216	<1.5 / 1.5-4 / >4	39 (89) / 5 (11) / 0	170 (96) / 6 (3) / 1 (1)	0.084
	Pre-Op		1.1 (0.9)	0.9 (0.5)	0.317
	n=230	<1.5 / 1.5-4 / >4	38 (84) / 7 (16) / 0	161 (89) / 19 (10) / 1 (1)	0.567
NLR	Pre-CRT		2.8 (2.3)	3.0 (2.2)	1.000
	n=222	<3 / ≥3	26 (58) / 19 (42)	107 (59) / 76 (41)	0.933
	Post-CRT		7.0 (11.5)	4.7 (3.0)	0.498
	n=216	<3 / ≥3	10 (23) / 34 (77)	26 (15) / 151 (85)	0.196
	Pre-Op		3.8 (3.6)	4.1 (3.4)	1.000
	n=230	<3 / ≥3	15 (33) / 30 (67)	59 (33) / 122 (67)	0.925
Hb (g/dL)	Pre-CRT		140 (24)	129 (20)	0.004
Categorical Hb	n=222	NO / YES	41(91) / 4(9)	135 (74) / 48 (26)	0.013
Hb <130g/dL (men) <115g/dL (women)	Post-CRT		130 (35)	125 (23)	0.001
	n=216	NO / YES	34 (77) / 10 (23)	102 (58) / 75 (42)	0.017
	Pre-Op		136 (23)	133 (20)	0.110
	n=230	NO / YES	39 (87) / 6 (13)	131 (73) / 49 (27)	0.052
Albumin (g/L)	Pre-CRT		37 (8)	37 (5)	0.148

	n=222	<35 / ≥35	8 (18) / 37 (82)	39 (21) / 143 (79)	0.588
	Post-CRT		35 (5)	36 (4)	0.065
	n=216	<35 / ≥35	11 (25) / 33 (75)	58 (33) / 119 (67)	0.320
	Pre-Op		35 (5)	37 (4)	0.022
	n=186	<35 / ≥35	8 (19) / 35 (81)	42 (24) / 134 (76)	0.461
CEA (µg/L)	Pre-CRT		2.8 (1.9)	3.7 (9.1)	0.114
	n=157	<5 / ≥5	28 (78) / 8 (22)	67 (57) / 50 (43)	0.027
	Post-CRT		2.2 (1.4)	3.4 (8.3)	0.022
	n=186	<5 / ≥5	29 (91) / 3 (9)	79 (62) / 48 (38)	0.002
CRP (mg/L)	Pre-CRT		3.0 (2.0)	3.5 (6.0)	0.895
	n=129	≤10 / >10	20 (91) / 2 (9)	86 (82) / 19 (18)	0.301
	Post-CRT		2.1 (4.0)	4.0 (5.0)	0.383
	n=186	≤10 / >10	31 (82) / 7 (18)	107 (73) / 40 (27)	0.267
	Pre-Op		1.7 (4.0)	4.0 (5.0)	0.573
	n=143	≤10 / >10	26 (90) / 3 (10)	93 (84) / 18 (16)	0.430
mGPS	Pre-CRT	0 / 1 / 2	20 (91) / 1 (4) / 1 (4)	86 (81) / 9 (9) / 10 (10)	0.585
	n=124				
	Post-CRT	0 / 1 / 2	31 (82) / 1 (3) / 6 (16)	107 (73) / 14 (9) / 26 (18)	0.342

	n=175				
	Pre-Op n=133	0 / 1 / 2	26 (90) / 1 (3) / 2 (7)	96 (84) / 10 (9) / 8 (7)	0.606

WCC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein; mGPS modified Glasgow prognostic score

Temporal changes in inflammatory parameters and categories of treatment response.

Tumour regression grade

Table 2.8s and figures 2.5s (located within appendix) demonstrate trends in median values of serum markers across the 3 timepoints around CRT related to the degree of pathological response measured by tumour regression grade. Total white cell, lymphocyte and neutrophil counts fell immediately after CRT and increased by the pre-op timepoint comparably in both response groups. A similar pattern was seen for NLR. Median CRP reduced immediately after CRT with favourable response having a lower median CRP at all 3 time points most pronounced post-CRT but did not reach significance. Albumin falls during therapy but patients with a good response have a higher serum albumin post-CRT and pre-operatively. The overall trends for changes in systemic inflammatory parameters were similar across TRG categories with no statistically significant divergence in changes between timepoints except for albumin.

NAR Score

Table 2.9s and figures 2.6s (located within appendix) demonstrate no significant differences in total white cell count, lymphocyte count, neutrophil count and NLR with minimal differences related to a NAR score <8 / ≥8. Median CRP was higher in at all timepoints with an unfavourable NAR (≥8) but not statistically significant. The statistical difference in albumin count between NAR groups does not translate into clinical difference. The overall trends for changes in systemic inflammatory parameters were similar across NAR categories with no statistically significant divergence in changes between timepoints except for albumin.

Pathological Complete Response

Table 2.10s and figures 2.7s (located within appendix) demonstrate similar trends in total white cell count, lymphocyte count, neutrophil count and NLR with no significant differences in the presence of pathological complete response. Median CRP was higher at all 3 timepoints in those without complete pathological response but was not statistically significant. Albumin again is higher post-CRT and pre-operatively in those with a complete pathological complete response. The overall trends for changes in systemic inflammatory parameters were similar across pCR categories with no statistically significant divergence in changes between timepoints except for albumin.

Binary logistic regression analysis for neoadjuvant treatment response.

In order to determine the most important parameters associated with response to treatment, a binary logistic regression analysis was performed to include pre-treatment clinical characteristics. Tables 2.4.1-3 detail this binary logistic regression analysis for the association between pre-treatment clinical characteristics, serum markers and treatment response measured using tumour regression grade (Table 2.4.1), NAR score (Table 2.4.2) and pathological complete response (Table 2.4.3). On univariate analysis, only larger tumour size (>8cm), elevated pre and post-treatment CEA and pre and post treatment anaemia were associated with poorer tumour regression (TRG 2/3). On multivariate analysis, only post treatment CEA ($\geq$5) was independently associated with poor tumour response.

On univariate analysis, pre and post treatment elevated CEA and pre-treatment anaemia were associated with high NAR score (surrogate marker of poor prognosis). On multivariate analysis only pre-treatment CEA and anaemia were associated with a high NAR score.

On univariate analysis pre and post treatment elevated CEA, and pre and post-treatment anaemia were associated lower rates of pathological complete response (pCR). On multivariate analysis only pre-treatment CEA and anaemia were independently associated with lower rates of pCR.

Table 2.4.1 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors and tumour regression grade (TRG). Odds ratio and 95% confidence interval in parenthesis.

		TRG 2/3 (poor) vs 0/1 (good) OR (95% CI)			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
Age	<55	1			
	55-75	0.99(0.48-2.02)	0.974		
	>75	0.50(01.88-1.34)	0.168		
BMI n=182	<25	1			
	25-30	2.16 (1.04-4.52)	0.040	1.64 (0.50-5.40)	0.417
	>30	1.28 (0.56-2.91)	0.556		
Tumour Height from anal verge (cm)	<5	1			
	5-10	1.28 (0.68-2.39)	0.441		
	>10	0.83 (0.39-1.81)	0.644		
Tumour Size (cm)	<4,	1			
	4-7	0.62(0.29-1.32)	0.212		
	≥8	0.21(0.06-0.76)	0.017	0.31 (0.05-1.91)	0.206
Clinical T Stage	T2	1			
	T3	1.52(0.44-5.23)	0.510		
	T4	0.30(0.07-1.39)	0.303		
Clinical N Stage	N0	1			
	N1&2	0.94(0.51-1.74)	0.850		
MRI EMVI n=106	No	1			
	Yes	0.73(0.31-1.72)	0.471		
Interval: CRT finish to Surgery (weeks)	<8	1			
	8-12	1.65(0.64-4.28)	0.301		
	>12	1.29(0.47-3.53)	0.618		
Pre-treatment CEA (µg/L) n=157	<5	1			
	≥5	0.38(0.19-0.78)	0.008	1.24 (0.19-7.93)	0.823
	<5	1			

Post-treatment CEA (μg/L) n=216	≥5	0.31(0.14-0.69)	0.004	0.40 (0.15-1.08)	0.045
Pre-treatment Anaemia <130g/dL (men) <115g/dL (women)	No	1			
	Yes	0.35(1.11-0.55)	<0.001	0.63 (0.18-2.24)	0.473
Post-treatment Anaemia n=216	No	1			
	Yes	0.41(0.22-0.77)	0.005	0.52 (0.20-1.34)	0.190
Pre-treatment NLR	<3	1			
	≥3	0.79(0.45-1.28)	0.399		
Post-treatment NLR n=216	<3	1			
	≥3	0.64(0.31-1.32)	0.226		
Pre-treatment mGPS	0	1			
	1	0.45(0.09-2.21)	0.323		
	2	0.40(0.08-1.93)	0.252		
Post-treatment mGPS n=157	0	1			
	1	0.59(1.18-1.98)	0.391		
	2	0.54(0.22-1.29)	0.164		

TRG AJCC tumour regression score quantifies tumour regression to neoadjuvant chemoradiotherapy; NAR Neoadjuvant rectal score; pCR pathological complete response; BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; mGPS modified Glasgow Prognostic Score.

Table 2.4.2 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors and NAR score. Odds ratio and 95% confidence interval in parenthesis.

		NAR score >8 (poor / intermediate) vs <8 (good) OR (95% CI)			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
Age	<55	1			
	55-75	1.35(0.59-3.06)	0.480		
	>75	0.69(0.22-2.14)	0.516		
BMI n=182	<25	1			
	25-30	2.15(0.92-4.99)	0.076		
	>30	1.13(0.42-3.06)	0.811		
Tumour Height from anal verge (cm)	<5	1			
	5-10	1.20(0.60-2.41)	0.607		
	>10	1.04(0.44-2.45)	0.930		
Tumour Size (cm)	<4,	1			
	4-7	0.79(3.35-1.78)	0.564		
	≥8	0.24(0.05-1.21)	0.084		
Clinical T Stage	T2	1			
	T3	5.22(0.67-40.82)	0.115		
	T4	1.56(0.16-15.16)	0.704		
Clinical N Stage	N0	1			
	N1&2	0.60(0.31-1.15)	0.124		
MRI EMVI n=106	No	1			
	Yes	0.80(0.29-2.21)	0.673		
Interval: CRT finish to Surgery (weeks)	<8	1			
	8-12	1.82(0.58-5.70)	0.302		
	>12	1.31(0.39-4.40)	0.660		
	<5	1			

Pre-treatment CEA (µg/L) n=157	≥5	0.33(0.14-0.75)	0.008	0.27(0.10-0.76)	0.017
Post-treatment CEA (µg/L) n=216	<5	1			
	≥5	0.26(0.09-0.71)	0.009	0.84(0.11-6.24)	0.866
Pre-treatment Anaemia <130g/dL (men) <115g/dL (women)	No	1			
	Yes	0.22(0.08-0.65)	0.006	0.16(0.03-0.72)	0.017
Post-treatment Anaemia n=216	No	1			
	Yes	0.55(0.28-1.08)	0.086		
Pre-treatment NLR	<3	1			
	≥3	1.03(0.53-2.00)	0.933		
Post-treatment NLR n=216	<3	1			
	≥3	0.59(0.26-1.33)	0.200		
Pre-treatment mGPS	0	1			
	1	0.48(0.06-3.99)	0.495		
	2	0.43(0.05-3.56)	0.430		
Post-treatment mGPS n=157	0	1			
	1	0.25(0.03-1.95)	0.184		
	2	0.80(0.30-2.11)	0.647		

TRG AJCC tumour regression score quantifies tumour regression to neoadjuvant chemoradiotherapy; NAR Neoadjuvant rectal score; pCR pathological complete response; BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; mGPS modified Glasgow Prognostic Score.

Table 2.4.3 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors and pathological complete response (pCR). Odds ratio and 95% confidence interval in parenthesis.

		pCR No vs Yes OR (95% CI)			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
Age	<55	1			
	55-75	1.29 (0.54-3.04)	0.567		
	>75	0.64 (0.19-2/16)	0.474		
BMI n=182	<25	1			
	25-30	1.65 (0.71-3.82)	0.246		
	>30	0.87 (0.31-2.40)	0.784		
Tumour Height from anal verge (cm)	<5	1			
	5-10	0.37 (0.66-2.81)	0.400		
	>10	0.81(0.31-2.11)	0.670		
Tumour Size (cm)	<4,	1			
	4-7	0.64 (0.28-1.45)	0.281		
	≥8	0.11 (0.01-0.96)	0.046		
Clinical T Stage	T2	1			
	T3	4.63 (0.59-36.26)	0.144		
	T4	0.36 (0.02-6.13)	0.479		
Clinical N Stage	N0	1			
	N1&2	0.57 (0.29-1.13)	0.108		
MRI EMVI n=106	No	1			
	Yes	0.56 (0.18-1.7)	0.318		
Interval: CRT finish to Surgery (weeks)	<8	1			
	8-12	3.22(0.72-14.50)	0.128		
	>12	2.88(0.61-13.57)	0.182		
Pre-treatment CEA (µg/L) n=157	<5	1			
	≥5	0.38 (0.16-0.91)	0.030	0.21(0.07-0.68)	0.009

Post-treatment CEA (µg/L) n=216	<5	1			
	≥5	0.17(0.05-0.59)	0.005	0.52(0.06-4.49)	0.549
Pre-treatment Anaemia <130g/dL (men) <115g/dL (women)	No	1			
	Yes	0.27(0.09-0.81)	0.019	0.29(0.06-1.40)	0.047
Post-treatment Anaemia n=216	No	1			
	Yes	0.04(0.19-0.86)	0.019	0.99(0.31-3.15)	0.987
Pre-treatment NLR	<3	1			
	≥3	0.93(0.50-1.75)	0.831		
Post-treatment NLR n=216	<3	1			
	≥3	0.61(0.28-1.34)	0.217		
Pre-treatment mGPS	0	1			
	1	0.38(0.05-3.15)	0.370		
	2	0.34(0.04-2.81)	0.317		
Post-treatment mGPS n=157	0	1			
	1	0.21(0.03-1.66)	0.139		
	2	0.68(0.26-1.79)	0.433		

TRG AJCC tumour regression score quantifies tumour regression to neoadjuvant chemoradiotherapy; NAR Neoadjuvant rectal score; pCR pathological complete response; BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; mGPS modified Glasgow Prognostic Score.

Changes in circulating measures of systemic inflammation pre and post-neoadjuvant treatment and their association with response.

In total, 221 evaluable patients had circulating measures of SIR at baseline and following initiation of neoadjuvant radiotherapy: 221 patients had white cell count and 109 patients had CRP measurements at baseline and following CRT. Figure 2.2 demonstrates median total white cell count, neutrophil count, lymphocyte count, and albumin fell following neoadjuvant CRT and recovered preoperatively in 144 patients who with blood results available at all 3 time points. Neutrophil to lymphocyte ratio (NLR) increased following CRT and fell pre-operatively. CRP increased following CRT and dropped pre-operatively, but this was not significant. In general, as detailed in Table 2.2, there is fall in these parameters after CRT with recovery by the pre-surgery time point. The Table 2.5 demonstrates the largest of these falls proportionally was in lymphocyte counts which fell by 61% from baseline to post-CRT and 50% from baseline to pre-operatively. This is likely to the main contributor to the NLR rising by 76% from baseline to post-CRT and 47% from baseline to pre-operatively. There was no significant change in median CRP from baseline to post-CRT but there was a numerical fall of 33% from baseline to pre-operatively.

Table 2.5 Median change in blood parameters between baseline to following neoadjuvant therapy and from baseline to pre-operatively. Percentage changes in parenthesis.

	Absolute Change between time points	
	Baseline to post CRT	Baseline to pre-op
WBC (10^9L) n=221	-2.5 (−35)	-2.1 (−28)
Neutrophils (10^9L) n=221	-1.4 (−30)	-0.9 (−20)
Lymphocytes (10^9L) n=221	-1.1 (−61)	-0.9 (−50)
NLR n=221	1.8 (+75)	1.3 (+47)
CRP (mg/L) n=109	0.0 (0)	-0.9 (−33)
Albumin (g/L) n=220	-2.0 (−5)	0.0 (0)
CEA (μg/L) n=112	-0.3 (−9)	NA

CRT chemoradiotherapy; WBC total white cell count; NLR neutrophil to lymphocyte ratio; CEA carcinoembryonic antigen; CRP C-reactive protein.

Figure 2.2.1 White Cell count (WCC) over time

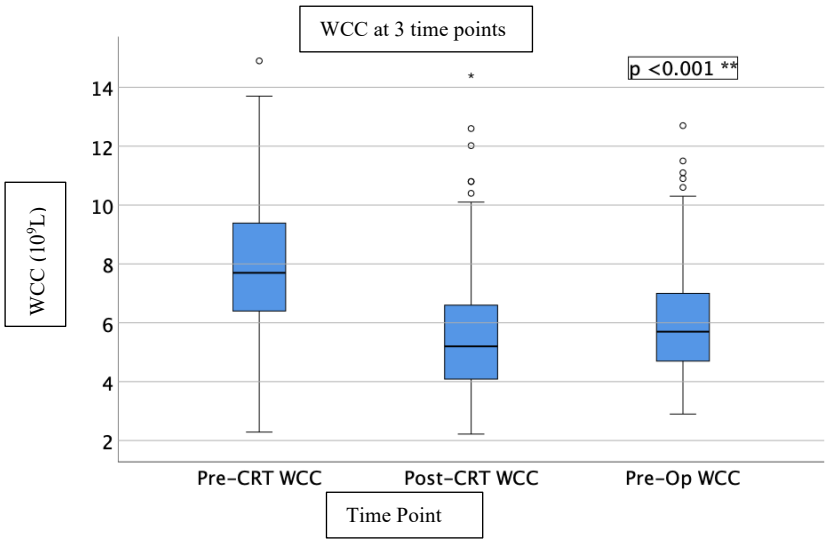


Figure 2.2.2 Lymphocyte count over time

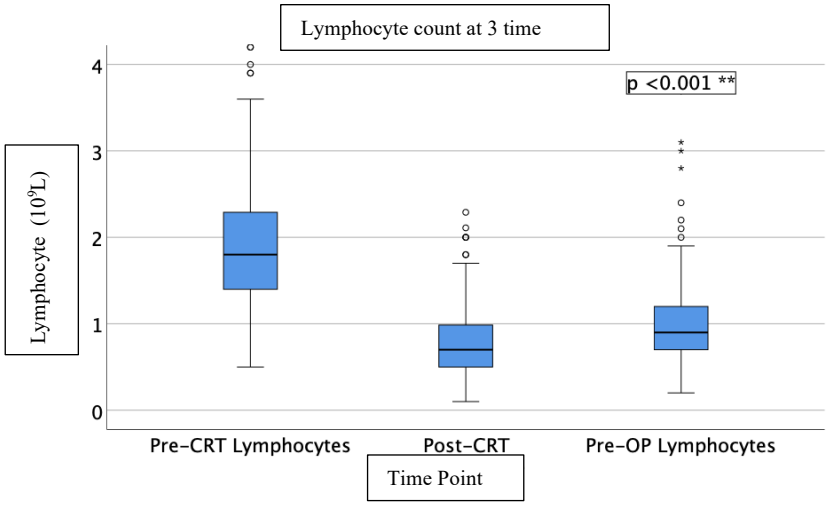


Figure 2.2.3 Neutrophil count over time

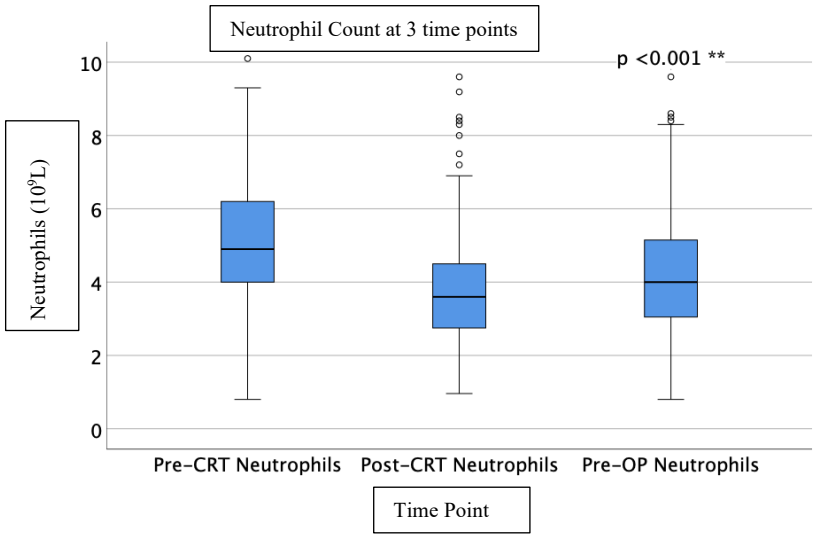


Figure 2.2.4 Neutrophil to lymphocyte ratio (NLR) over time

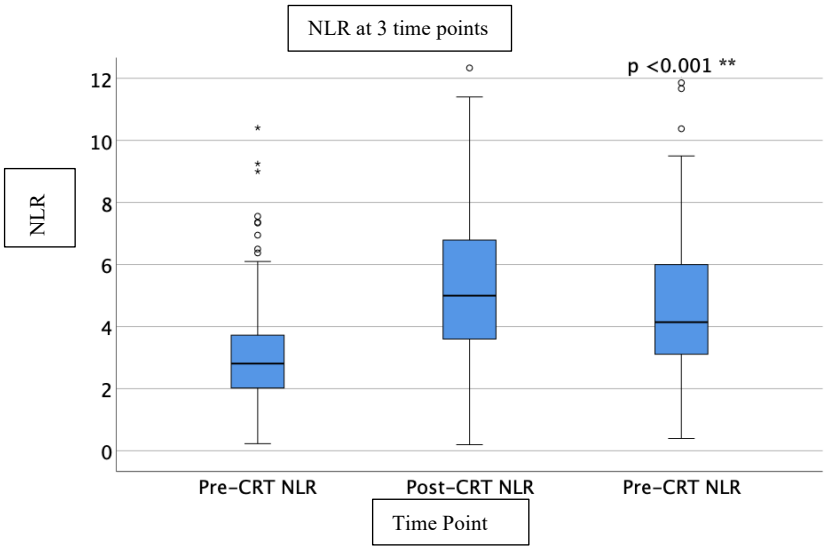


Figure 2.2.5 C-Reactive protein (CRP) over time

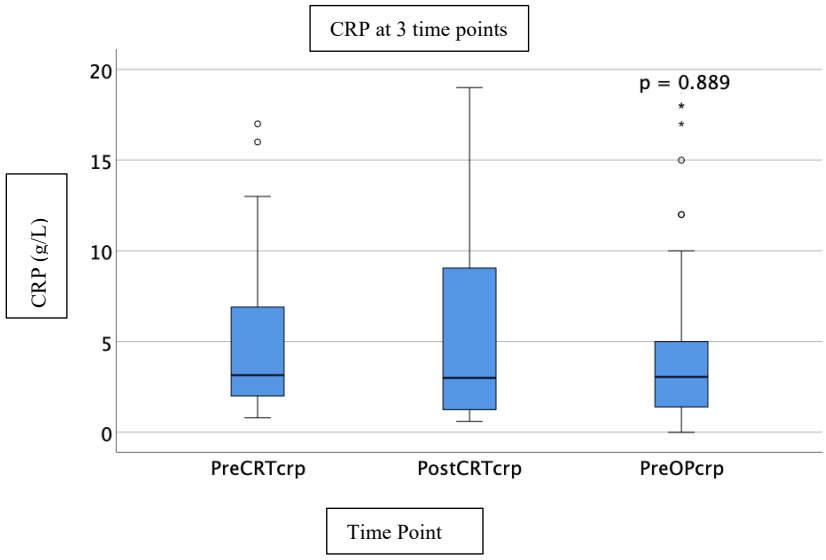
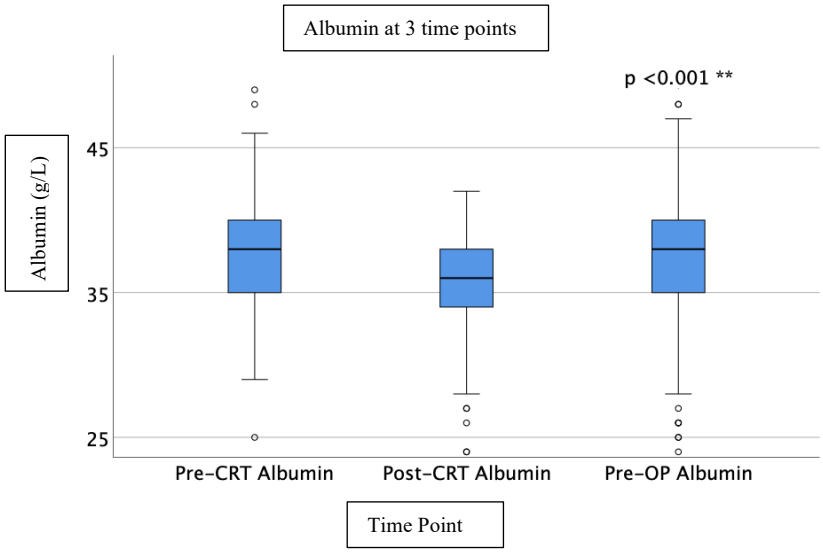


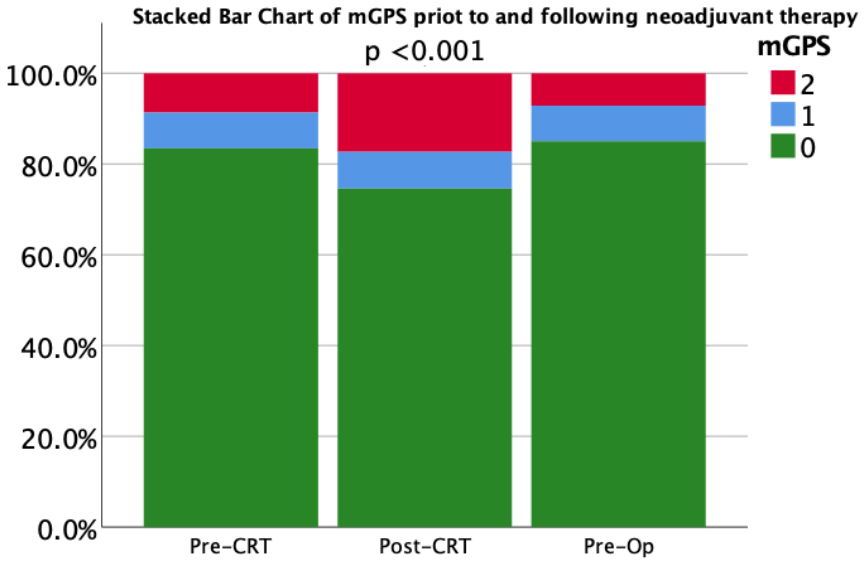
Figure 2.2.6 Albumin over time



Box plot of serum markers prior to and following neoadjuvant therapy. P value denotes Kruskal Wallis testing for difference in medians between 3 time points.

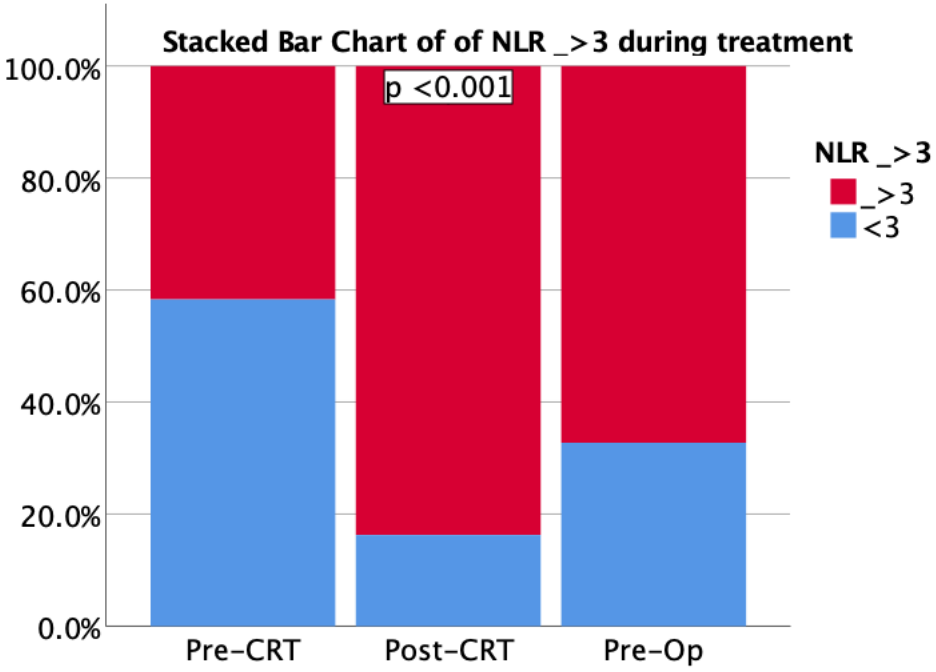
Figures 2.3 shows the proportion of patients with an elevated mGPS increased immediately following CRT and returns to near baseline pre-operatively. The proportion of patients with an mGPS of 1 appears static across time points. Those with a mGPS of 2 increases during CRT and returns to near baseline preoperatively. Figure 2.4 shows the proportion of patients with an elevated NLR ≥3 increases immediately following CRT and remains higher pre-operatively compared to baseline (Figure 2.4). In Table, 2.7, the temporal changes in systemic inflammatory parameters are considered as categorical variables (e.g. fall, rise, static etc, as defined in the methods section above). The proportion of patients with an elevated NLR increases from low to high in 43% of patients from baseline to post-CRT and stays high in 41%. It increases from low to high in 34% from baseline to pre-op and stays high in 33%. It falls from high to low in only 1% from baseline to post-CRT and 8% from baseline to pre-operatively.

Figure 2.3 mGPS prior to and following neoadjuvant therapy.



Stacked bar chart modified Glasgow Prognostic Score (mGPS) prior to and following neoadjuvant therapy. P value denotes Chi squared testing.

Figure 2.4 NLR <3 or ≥3 prior to and following neoadjuvant therapy



Stacked bar chart of NLR greater than or less than 3 prior to and following neoadjuvant therapy. P value denotes Chi squared testing.

Table 2.6 Change in CRP, NLR, neutrophil, lymphocyte, white cell counts prior to and following neoadjuvant therapy. Numbers in parenthesis indicate percentages.

		Baseline to Post-CRT (%)	Baseline to Pre-Op (%)
WCC	Fall	130 (59)	106 (47)
	Rise	5 (2)	4 (2)
	Static	86 (39)	115 (51)
		n=221	n=225
Neutrophils	Fall	76 (34)	55 (24)
	Rise	11 (5)	9 (4)
	Static	134 (61)	162 (72)
		n=221	n=226
Lymphocytes	Fall	163 (74)	116 (51)
	Rise	1 (1)	2 (1)
	Static	57 (26)	108 (48)
		n=221	n=226
NLR	Fall	0	3 (1)
	Rise	35 (16)	30 (13)
	Static	186 (84)	193 (85)
		n=221	n=226
NLR High >3 Low <3	Low - Low	33 (15)	55 (24)
	Low - High	94 (43)	77 (34)
	High - High	91 (41)	75 (33)
	High - Low	3 (1)	19 (8)
		n=221	n=225
CRP	Low - Low	77 (71)	74 (80)
	Low - High	15 (14)	5(5)

	High - High	5 (5)	8 (9)
	High - Low	12 (11)	5 (5)
		n=109	n=92

CRT chemoradiotherapy; WBC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein.

Table 2.11s (located in the appendix) demonstrates most patients developed a high NLR (≥3) or had an NLR which stayed from baseline to following CRT and from baseline to pre-operatively irrespective of the pathological response measured by pathological tumour regression. CRP remained low for the majority (74% and 68%) of patents in tumour response groups from baseline to following CRT. Poor responders had a higher proportion of patients starting with a high CRP which stayed high or fell to normal during therapy, but this did not reach statistical significance. This pattern was similar from baseline to pre-operatively. When the no inflammation at baseline group (no inflammation throughout or development of inflammation during treatment) and inflammation at baseline group (inflammation throughout or loss of inflammation during treatment) from baseline to following CRT are compared, 6.1% in the low group had a poor repones compared 19% of the high CRP group (p=0.05). mGPS remained low in the majority of patients from baseline to following CRT and baseline to preoperatively at 74% and 82% respectively in those with a good response and 68% and 75% respectively in those with a poor response. A greater proportion of patients with a good response had an increase in mGPS from baseline to post-CRT (28% vs 12%) but this did not reach statistical significance and was not observed from baseline to pre-operatively. Changes in mGPS was not associated with the degree of response.

Table 2.12s (located in the appendix) demonstrates no significant trends in NLR during both time intervals irrespective of NAR score. CRP remained low from baseline to following CRT in 76% and 69% of patients with a low and high NAR score respectively. CRP also remained low from baseline to pre-operatively for 90% and 78%, for low and high NAR score respectively but did not reach significance. 5% of patients with a low NAR score had a high CRP which stayed high or fell from baseline to following CRT. This was not significant but no patients with a low NAR score had a high CRP which stayed high or fell between baseline and pre-operatively, the latter was significant (p=0.047). mGPS remained low between baseline and post-CRT 76% with a low NAR score and 70% with a high NAR score. mGPS remained low between baseline and pre-operatively 90% with a low NAR score and 75% with a high NAR score. Only 5% of patients with a high mGPS which stayed high or fell from baseline to following CRT had a low NAR. This was more pronounced with no patients in these groups from baseline to pre-operatively having a low NAR score but neither reached significance.

Table 2.13s (located in the appendix) demonstrates NLR changes were similar from baseline to following CRT and baseline remained low across both time intervals in the majority of patients irrespective of pCR. No patients who had a pathological complete response had a starting high CRP, but this did not reach statistical significance (p=0.064). mGPS increased from baseline to following CRT in a 28% of those with a pCR compared to 12% in those without a pCR but did not reach significance (p=0.145). This was not observed between baseline and pre-operatively.

2.4 Discussion

The aim of this chapter was to investigate the relationship between biomarkers of systemic inflammation at 3 time points around treatment and response to neoadjuvant therapy. My secondary aim was to investigate if temporal changes in these parameters were associated with treatment response. This was a large cohort of neoadjuvant therapy treated rectal cancer patients and given 20% of patients had pathological complete response (pCR) the cohort is reflective of a standard locally advanced rectal cancer population [220]. Only 5% patients had a complete clinical response and were treated non-operatively. This is reflective of a time where the non-operative management and organ preservation reserved for patients were unsuitable for surgery or did not wish to have surgery. I hypothesised that measures of systemic inflammation would be consistently associated with poorer treatment response. Despite a thorough analysis, I did not observe a strong association between pre or post treatment measures of systemic inflammation and treatment response. I did validate the prognostic role of tumour size, pre and post treatment CEA and anaemia in terms of treatment response. In addition, I have observed consistent changes in circulating parameters during treatment and after treatment.

During treatment total white cell count, neutrophil count, lymphocyte count, haemoglobin and albumin decreased following treatment and recovered but not back to baseline preoperatively. CEA decreased during treatment. NLR and CRP increased during treatment as did the proportion of patients with an elevated CRP and mGPS.

There were no changes in serum biomarkers which were associated with improved tumour response / favourable NAR or pathological complete response.

In this study WCC, lymphocyte count, NLR, CRP, mGPS were not associated with tumour regression grade which differs from a previous negative association between an elevated

mGPS and elevated NLR being associated with poorer tumour response. [211] In the Dreyer et al study, there were fewer patients and from an earlier time point where assessment tumour response grading was variable prior to the implementation of standardised pathology reporting. [221] A strength of our study is the evaluating associations with 3 separate measures of treatment response, which take into account tumour regression, down staging and complete responses. Another difference between then Dreyer et al study and my current study is the higher clinical node positivity, higher numbers of clinical stage III patients and that more patients waited >12 weeks for surgery from the end of neoadjuvant therapy in my study. To the best of our knowledge, our study is the first to investigate the relationship between circulating markers of systemic inflammation and the NAR score.

Characteristics with an independent association with tumour response were CEA; anaemia; and tumour size. This is in keeping with previous studies which have demonstrated smaller tumour size and low post treatment CEA are important determinants of complete clinical and pathological response. [222] [223] Other groups have found using area under curve analysis that high lymphocyte to CRP ratio; high neutrophil x lymphocyte counts were associated with good pathological response. This combined with high CD8 tumour infiltrating lymphocyte (TIL) counts were independently associated with an improved response. Sawada et al found there was no correlation between the circulating lymphocyte count and tumour CD8 TIL counts, and did not assess NLR influence on response [224].

Our study demonstrated lower baseline NLR was associated with poorer NAR score. This is likely a spurious result as multiple comparisons were made in this analysis so the

potential for a small number of spurious results exists. These results would be strengthened if there were consistent relationships seen across more measures of response and more than one or two inflammatory parameters. High baseline NLR has been associated poorer survival on metanalysis but there was significant difference in NLR cut off values. [225] Elevated post treatment NLR (>3.23) was independently associated with lower rates of pCR and elevated baseline NLR (>2.77) was independently associated with poorer RFS. However, the cut-offs used by Jeon et al, like many have been determined by ROC analysis rather that more widely accepted values. Jeon et al also identified the following as negative predictors of pCR: being clinical node positive; elevated CEA; baseline NLR>2.77; post-treatment NLR >3.23 [226] Meanwhile other studies have demonstrated improved survival but no difference in pCR with an NLR cut off of 2.3. [227] This highlights the inconsistencies in the literature due to heterogenous cohorts with varying tumour size, distance from anal verge, dose of chemoradiation and interval from completion of CRT to surgery. Mechanisms behind high NLR and poorer tumour response / survival in rectal cancer are not understood. Patients with high NLR have been observed to have higher circulating pro-inflammatory cytokines which result in a tumour microenvironment with more aggressive tumour growth. Interleukins 1 receptor antagonists protein, 6, 7, 8, 12, 17, monocyte chemoattractant protein (MCP-1) and platelet derived growth factor BB (PDGFBB) have been implicated in patients with a higher circulating NLR. These elevated cytokine levels are associated with increased tumour macrophage infiltration and an upregulated innate immune system. [170] [228] [229].

This chapter's results demonstrate consistent changes in NLR increasing from the baseline to post-CRT and then falls slightly preoperatively. This fall is largely as a result of a greater fall in lymphocyte counts. The proportion of patient with an elevated mGPS also increased during treatment. Despite lower CRP trending towards improved treatment

response, this did not reach significance. Albumin fell during treatment but patients with a higher albumin post -CRT being associated a good response (TRG0/1) and complete pathological response. This is reflective of previous studies which have found albumin >35 is associated with pCR but more so in stage III disease. [215] Hypoalbuminaemia is a recognised marker of poor prognosis as part of the modified Glasgow Prognostic Score in patients with colorectal and other cancers. Hypoalbuminaemia is likely to reflect the ongoing systemic inflammatory response. [230] [231] [232]

There was no association identified between changes in the total white cell count, its constituents, NLR and treatment response. In other studies, assessing changes in NLR, ROC curves were used to determine an optimal cutoff of 21.5% change from baseline to pre surgery. Lai et al found NLR change of ≤21.5% was associated with higher rates of pCR. In this cohort nearly a third of patients had oxaliplatin based chemotherapy, independently associated with pCR, considerably higher than in our study. [233] NLR is a reflection of the balance between pro-tumour and anti-tumour responses. Elevated NLR could be as a result of the pro-tumour neutrophil activity as the rise in NLR observed in our study was predominantly from a fall in lymphocytes.

Lower rates of pCR (11 vs 37%) were observed in those with a CEA remaining consistently high from baseline to post CRT. 85% of those with pCR and 81% of those with NAR <8 had a consistently low CEA. This is consistent with previous studies which have demonstrated improved pCR with normal pre-treatment CEA. As elevated CEA has been associated with larger tumours which have lower rates of pCR, CEA is likely a representation of disease burden. [156, 234-236]

In my study CEA was only measured at baseline and post-CRT, with no repeat pre-surgery. Most previous studies have assessed CEA at baseline or pre-operatively. To our best knowledge this is the first study to assess changes in CEA during treatment. In our

study we do not have the smoking status which has been associated with mild increases in CEA. [236]

Good tumour regression and NAR <8 was associated with low CRP at baseline, or which increased from low to high during treatment suggesting these patients were not systemically inflamed at baseline but developed this during the course of treatment. This trend did not reach significance when observing changes from baseline to pre surgery indicating a level of inflammation in all patients. CRP which started low and remained low or increased following therapy was associated with lower rates of poor response (TRG 2/3) compared to patients who started with a high CRP.

This study combined 2 cohorts of patents treated at different time points with differences in clinical TNM staging and time interval between neoadjuvant therapy and surgery. Just over half of the patients in our cohort undergoing neo-adjuvant therapy for rectal cancer underwent sphincter excising TME resections indicating the possibility of more advanced disease. Having patients from 2008-2014 there would be variations in local reporting of staging MRI scans across multiple units before standardised reporting was commonplace. [237] This study used retrospective collection of clinicopathological data with tumour regression grade not always clearly reported. If resources and time allowed reassessment of tumour response by a specialist pathologist would have overcome this, however one positive to this methodology could be the fact that these results are representative of real world data in routine care. Another limitation to this study was the timing of blood sampling (which were selected as close to the study time points) and lower number of patients with CRP data. This introduces variability in timing and thus type 2 error. With a larger sample size, it would have been informative to study changes in CRP and mGPS from baseline to post-CRT and then pre-operatively to have a 3 point trend. Systemic inflammation can also be affected by concurrent infective pathology. Previous studies have had been heterogeneity in the classification of treatment response. One of the main

difficulties encountered by studies measuring circulating markers of systemic inflammation is the selection of which markers to assess. This study in its retrospective nature only had access to routine serum makers such as the differential full blood count and biochemistry.

In conclusion, I observed that there is variation in measures of the systemic inflammatory response across three time points before, during and after neoadjuvant therapy. There was no apparent relationship between components of the SIR and SIR-based prognostic scores and treatment response to radiation at each time point. I observed an increase in NLR from baseline to post-CRT then falling slightly preoperatively. This was due to a predominant fall in the lymphocyte count during chemoradiotherapy. Treatment related lymphopenia has been reported as a potential biomarker for response to neoadjuvant therapy which will be further assessed in Chapter 3. CRP which remained low or fell during treatment was associated with a favourable NAR score but there was no association between changes in NLR, CRP or mGPS on tumour response or pCR. Albumin which remained high (≥35) following treatment was associated with improved tumour regression and pCR. CEA which remained high after treatment was associated with lower rates of pCR and unfavourable NAR score. I did however observe consistent relationships between haemoglobin level and CEA level and treatment response. These measures may relate to tumour size and burden, but anaemic patients may have lower tissue oxygen levels which could impair RT responses. Anaemia and its impacts on treatment response in rectal cancer will be further investigated in Chapter 4.

3 An investigation into the influence of lymphopenia on response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer

3.1 Introduction

Lymphopenia is commonly seen following radiotherapy in a variety of solid tumour types. [185]. In reviews by Menetrier-Caux et al and Venkatessulu et al, lymphopenia at baseline was associated in poorer progression free survival and disease free survival over a number of solid tumours. Irradiation of lymphopoietic regions like the pelvis (lumbosacral bone marrow, iliac vessels, pelvic lymph nodes) may contribute to lymphopenia as will irradiation of tumour infiltrating lymphocytes within the irradiated field. These studies were mainly in non-rectal cancer patients. [238] [185] Camian et al demonstrated 35% of patients with rectal cancer undergoing CRT have grade III / IV lymphopenia at 2 months with lymphopenia persisting up to 12 months from treatment. [186] Higher peripheral lymphocyte counts during neoadjuvant therapy have been associated with improved treatment response. Heo et al demonstrated that total white cell counts, and its subtypes fell during treatment and recovered afterwards. Despite baseline nor absolute counts being associated with pCR a sustained lymphocyte ratio of ≥ 0.35 was associated with pCR, hypothesising this could be a maker of the maintenance of the host immunity during chemoradiotherapy. [187] Despite improved tumour response from tumour infiltrating T cells, this has not shown to be directly correlate with circulating lymphocyte counts. Flow cytometry by Schmidt et al has demonstrated an increase in dendritic cells in patients with colorectal and breast cancer compared to controls. Kitayma et al demonstrated a baseline lymphocyte count of $\leq 25.7\%$ was associated with higher rates pathological complete response. Higher lymphocyte count was also associated with improved overall and disease

free survival. They also showed the largest fall of white cell subtypes to fall was lymphocytes. [239]. A smaller study of Grade 2 vs 3-4 lymphopenia by Howel et al showed no difference in baseline characteristics, overall survival or NAR score but higher grade lymphopenia was associated with a poorer 2 year progression free survival. [240]

Having observed significant reduction in lymphocyte counts in previous chapters, the aim in this chapter was understand if treatment-related lymphopenia is associated with clinicopathological characteristics and also if treatment-related lymphopenia had a detrimental effect on treatment response. This chapter expands on the previous work with a specific focus on treatment-related lymphopenia categories enabling a more granular assessment than the routine thresholds applied in chapter 2. I hypothesise that more advanced tumours will be associated with increasing treatment-related lymphopenia (TRL) and a higher grade TRL will be associated with poorer response to neoadjuvant therapy.

3.2 Methods

The same patient database described earlier in systemic inflammatory response chapter was used to study the effect of pre-treatment and treatment-related lymphopenia on treatment response. Briefly, all patients had biopsy proven adenocarcinoma of the rectum with pre-treatment MRI and CT imaging to assess clinical TNM stage. Patients underwent radiotherapy dose was usually 45Gy in 25 fractions over 5 weeks with 3 patients receiving a boost to regional nodes (range 32-54Gy). Concomitant chemotherapy was oral capecitabine, intravenous 5-Flurourcail or capecitabine in combination with irinotecan or oxaliplatin.

Routine bloods tests taken in the 1-3 weeks before commencing CRT were defined as baseline bloods. Blood tests taken from the last week radiotherapy to 4 weeks after completing CRT were defined as post-treatment bloods. Blood tests taken 12-20 weeks

after commencing CRT and within 2 weeks of surgery were defined as pre-operative bloods. Blood investigations included full blood count incorporating a differential white cell count, routine biochemistry, C-reactive protein and carcinoembryonic antigen. All lab results were grouped according to standard thresholds. Lymphopenia following neoadjuvant CRT was graded based on severity as per CTCCAE v5 [241]. National Cancer Institute Common Terminology Criteria for Adverse Event (CTCAE) version 5 is the common terminology descriptors for adverse events related to medical treatments or procedures, in this case CRT. Categories were: no (Grade 0), mild (Grade 1), moderate (Grade 2), severe (Grade 3) and life threatening (Grade 4). These groups were dichotomised into Grade 0/1 and Grade 2+. Changes between the groups was studied from the post-CRT and pre-op time points. Neutrophil to Lymphocyte ratio was obtained from the differential blood count and modified Glasgow prognostic score (mGPS) was constructed using methods previously described in chapter 2. Briefly, patients were given 1 point for a serum CRP >10 mg/L and a further point if serum albumin <35g/L.

Neoadjuvant rectal (NAR) score, was calculated using clinicopathological parameters as a measure of long term survival, stratifying patients into low, intermediate and high risk.[157] MRI T or N downstaging was defined as a reduction in T or N stage on post treatment imaging compared to pre-treatment assessment.

Pathological tumour response from chemoradiotherapy was defined as per the Royal College of Pathologist colorectal cancer dataset based on the 4 tier system described by Ryan et al. [216, 217] Score of 0 if there were no viable cancer cells (complete response). Score 1 if there were single cells or small groups of cancer cells (near-complete response). Score 2 if residual cancer with evident tumour regression but more than single cells or small groups of cancer cells (partial response). TRG 3 if there is extensive residual cancer with no evidence of tumour regression (poor response). [217] Tumour response was

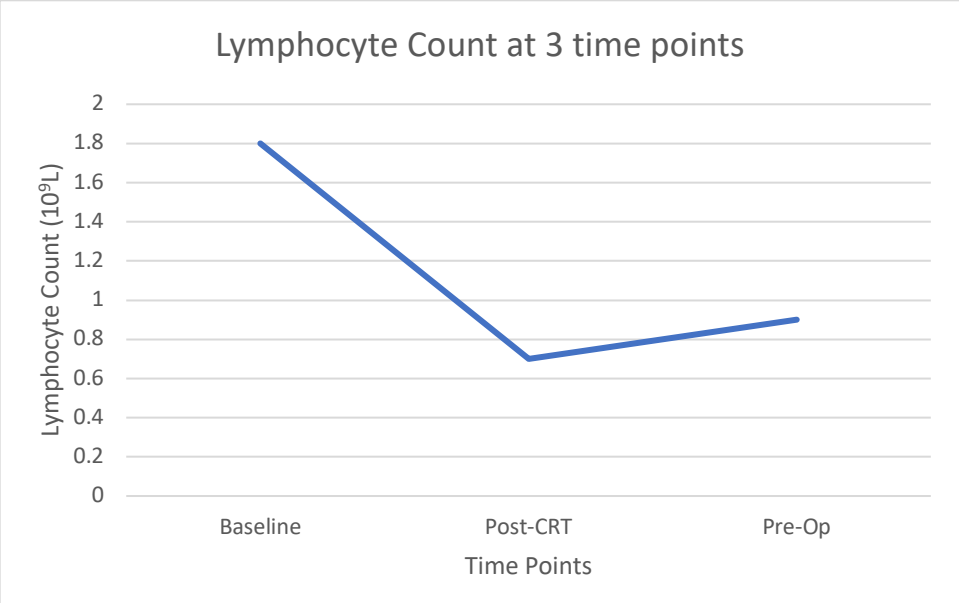
dichotomised into good / near complete response (TRG 0/1) and incomplete / poor response (TRG 2/3).

Statistical analysis was undertaken with using SPSS (version 25.0; IBM SPSS Statistics, IBM corp, Armonk, NY, USA). Variables were grouped using standard clinical thresholds or cut offs which have been established in literature. Comparison between groups was performed with the Chi square test, and Fisher's exact test was used where n was less than 5.

3.3 Results

228 patients who had nCRT were identified with differential white cell counts throughout treatment. As described in the previous chapter and displayed in Figure 3.1, the median lymphocyte count falling from baseline following CRT and recovering slightly. 91% patients had a normal lymphocyte count prior to starting therapy. Table 3.1 details this further with 8% of patients having baseline lymphopenia. Post-CRT only 19% had a normal lymphocyte count with 81% being lymphopenia. Post-CRT 26% had grade 1 lymphopenia and 55% had grade 2+ lymphopenia with only 1 patient having grade 4 lymphopenia. By the pre-operative time point 61% were lymphopenic and 39% had a normal lymphocyte count. From the post-CRT to pre-op time point the proportion of grade 1 lymphopenia increased from 26% to 35% whilst the proportion of patients with grade 2 or 3 lymphopenia fell from 55% to 26%.

Figure 3.1 lymphocyte count prior to and following CRT



Median lymphocyte count over time from baseline to following CRT and pre-op.

Table 3.1. Lymphocyte counts and lymphopenia grades prior to and following CRT. Numbers in parenthesis indicate percentages. P values for chi square testing.

		Pre CRT	Post CRT	Pre-OP	P value
Lymphocytes	low	19 (8)	179 (81)	138 (61)	<0.001
	Normal	208 (91)	41 (19)	87 (39)	
	High	1 (0.4)	1 (1)	1 (0.4)	
Lymphopenia	No	209 (92)	42 (19)	88 (39)	<0.001
	Grade 1	13 (6)	58 (26)	78 (35)	
	Grade 2	6 (3)	76 (33)	51 (23)	
	Grade 3	0	44 (20)	9 (4)	
	Grade 4	0	1 (0.4)	0	

Table 3.2 indicates the relationship between treatment related lymphopenia at the post-CRT / Pre-Op time points and markers of inflammation. There was a borderline association between grade 2+ lymphopenia and mild post-CRT anaemia, and the presence of no lymphopenia being associated with the absence of anaemia (p=0.058). There was no association between post-CRT lymphopenia and CRP or changes in mGPS. Post-CRT Lymphopenia G2+ trended towards an elevated mGPS but did not reach significance (p=0.167). There was no association between grade of lymphopenia and the grade of anaemia. The presence of pre-operative G2+ lymphopenia was associated with an elevation of CRP and mGPS or a consistently elevated CRP and mGPS baseline to pre-operatively (p=0.051). It was also associated with a lower proportion of patients with a CRP or mGPS which remained normal during this time (p=0.039). Absence of pre-operative lymphopenia was associated with normal mGPS (p=0.053).

Table 3.2 Relationship between lymphopenia and markers systemic inflammation. Percentages within parenthesis. P values indicate chi square testing.

		Post-CRT Lymphopenia			P value	Pre-Op Lymphopenia			P value
		No	G1	G2+		No	G1	G2+	
White cell count	<4	2 (45)	8 (14)	35 (29)	0.006	3 (3)	13 (17)	12 (20)	0.007
	4-11	39 (93)	50 (86)	84 (69)		83 (95)	65 (83)	46 (77)	
	>11	1 (2)	0	2 (2)		1 (1)	0	2 (3)	
NLR	<3	21 (50)	10 (17)	5 (4)	<0.001	64 (73)	8 (10)	2 (3)	<0.001
	≥3	21 (50)	48 (83)	116 (96)		24 (27)	70 (90)	58 (97)	
Anaemia	No	32 (76)	39 (67)	65 (54)	0.058	66 (76)	61 (78)	43 (72)	0.885
	Mild	8 (19)	14 (24)	48 (40)		19 (22)	16 (21)	15 (25)	
	Moderate	2 (5)	5 (9)	8 (7)		2 (2)	1 (1)	2 (3)	
Albumin	<35	10 (24)	16 (28)	43 (36)	0.267	68 (79)	50 (69)	51 (85)	0.068
	≥35	32 (76)	41 (72)	76 (64)		18 (21)	23 (31)	9 (15)	
CRP	≤10	31 (86)	37 (76)	68 (70)	0.167	50 (94)	37 (78)	32 (80)	0.053
	>10	5 (14)	12 (25)	29 (30)		3 (6)	10 (21)	8 (20)	
mGPS	0	31 (86)	37 (76)	68 (70)	0.139	50 (94)	37 (79)	32 (80)	0.165
	1	3 (8)	5 (10)	6 (6)		1 (2)	5 (11)	5 (13)	
	2	2 (6)	7 (14)	23 (24)		2 (4)	5 (11)	3 (8)	

Table 3.3 examines the relationship between the lymphopenia grade at the post-CRT and pre-operative time points and clinicopathological features. There were no significant associations between lymphopenia and age, gender, BMI, clinical stage, grade of response on MRI, lymphopenia and resection margin, EMVI or tumour differentiation. The absence of post-CRT lymphopenia trended towards a non-significant association with pathological ypT0 staging (31% vs 16-18%) p=0.073. There was increased proportion of pathological ypN2 in the absence of pre-operative lymphopenia but did not reach significance (p=0.090). There was no association between lymphopenia, radiation dose or type of chemotherapy.

Table 3.3. Relationship between lymphopenia and clinicopathological factors. Numbers in parenthesis indicate percentages. P values for chi square testing.

		Post-CRT Lymphopenia			p	Pre-Op Lymphopenia			p
		No	G1	G2+		No	G1	G2+	
Age (years)	<55	7 (17)	10 (17)	26 (22)	0.620	12 (14)	14 (18)	16 (27)	0.121
	55 – 74	27 (64)	34 (59)	77 (64)		55 (62)	51 (65)	38 (63)	
	≥75	8 (19)	14 (24)	18 (15)		21 (24)	13 (17)	6 (10)	
Sex	Female	17 (40)	22 (38)	47 (39)	0.967	34 (39)	29 (37)	24 (40)	0.944
	Male	25 (6)	36 (62)	74 (61)		54 (61)	49 (63)	36 (60)	
BMI	<25	13 (42)	16 (33)	40 (43)	0.272	28 (43)	26 (40)	15 (32)	0.726
	25-30	14 (45)	19 (40)	27 (29)		22 (34)	24 (37)	17 (36)	
	>30	4 (13)	13 (27)	26 (28)		15 (23)	15 (23)	15 (32)	
MRI	T2	5 (12)	5 (9)	5 (4)	0.360	7 (8)	4 (5)	3 (5)	0.588
	T3	32 (76)	42 (72)	92 (76)		65 (74)	58 (74)	50 (83)	
	T4	5 (12)	11 (19)	24 (2)		16 (18)	16 (21)	7 (12)	
MRI	N0	14 (33)	16 (28)	32 (26)	0.690	27 (31)	21 (27)	16 (27)	0.820
	N1/N2	28 (67)	42 (72)	89 (74)		61 (69)	57 (73)	44 (73)	
	<5 (low)	24 (59)	22 (39)	43 (38)	0.244	42 (48)	29 (41)	20 (35)	0.094

Tumour Height from anal verge (cm)	5 – 10 (mid)	11 (27)	23 (40)	45 (40)		31 (36)	22 (31)	28 (49)	
	>10 (upper)	6 (15)	12 (21)	24 (21)		14 (16)	20 (28)	9 (16)	
Tumour Size	<4	11 (27)	9 (16)	15 (13)	0.324	18 (21)	11 (15)	5 (9)	0.340
	4-7	27 (66)	42 (74)	88 (75)		61 (71)	55 (73)	46 (79)	
	≥8	3 (7)	6 (10)	14 (12)		7 (8)	9 (12)	7 (12)	
TNM Stage	I	2 (5)	1 (2)	2 (2)	0.770	2 (2)	2 (3)	1 (2)	0.970
	II	12 (29)	15 (26)	30 (25)		25 (28)	19 (24)	15 (25)	
	III	28 (67)	42 (72)	89 (74)		61 (69)	57 (73)	44 (73)	
MRI EMVI	No	8 (53)	14 (44)	16 (30)	0.190	10 (26)	18 (45)	10 (43)	0.161
	Yes	7 (47)	18 (56)	37 (70)		29 (74)	22 (55)	13 (57)	
MRI Response MRI Regression Grade	MR TRG 0/1	17 (55)	13 (36)	36 (47)	0.546	29 (50)	25 (50)	11 (31)	0.190
	MR TRG 2	11 (35)	20 (56)	36 (47)		23 (40)	22 (44)	23 (34)	
	MR TRG 3	3 (10)	3 (8)	5 (6)		6 (10)	3 (6)	23 (34)	
Pathological T staging	ypT0	13 (31)	9 (16)	22 (18)	0.073	20 (23)	17 (22)	8 (13)	0.268
	ypT1	0	2 (3)	9 (7)		3 (3)	5 (6)	4 (7)	
	ypT2	5 (12)	16 (28)	17 (14)		16 (18)	11 (14)	13 (22)	
	ypT3	22 (52)	29 (50)	62 (51)		46 (52)	36 (46)	33 (55)	

	ypT4	2 (5)	2 (3)	11 (9)		3 (3)	9 (11)	2 (3)	
Pathological N staging	ypN0	31 (74)	38 (65)	89 (74)	0.664	63 (72)	55 (71)	44 (73)	0.090
	ypN1	7 (17)	16 (28)	24 (20)		14 (16)	20 (26)	14 (23)	
	ypN2	4 (10)	4 (7)	8 (7)		11 (12)	3 (4)	2 (3)	
Resection Margin	R0	35 (83)	51 (88)	106 (88)	0.750	76 (86)	68 (87)	53 (88)	0.940
	R1 (≤1mm to CRM)	7 (17)	7 (12)	15 (12)		12 (14)	10 (13)	7 (12)	
EMVI	No	26 (68)	37 (67)	91 (78)	0.216	59 (71)	54 (73)	46 (81)	0.419
	Yes	12 (32)	18 (33)	25 (22)		24 (29)	20 (27)	11 (20)	
Tumour differentiation	Well, / Moderate	35 (95)	46 (90)	103 (92)	0.754	70 (90)	66 (93)	52 (93)	0.727
	Poor	2 (5)	5 (10)	9 (8)		8 (10)	5 (7)	4 (7)	
Type of concurrent chemotherapy	Capecitabine	23 (88)	40 (83)	82 (89)	0.435	57 (81)	47(87)	45 (96)	0.258
	5-FU	2 (8)	8 (17)	8 (9)		11 (16)	6 (11)	2 (4)	
	other	1 (4)	0	2 (2)		2 (3)	1 (2)	0	
Radiotherapy dose	<45Gy	0	2 (4)	6(5)	0.573	4 (5)	1 (2)	3 (6)	0.541
	45Gy	37 (100)	46 (94)	101 (93)		72 (92)	65 (97)	50 (94)	
	>45Gy	0	1 (2)	2 (2)		2 (3)	1 (2)	0	

Table 3.4 demonstrates the relationship between lymphopenia and markers of treatment response. Higher number of pCR were observed in the absence of post-CRT lymphopenia but this did not reach significance. Absence of Pre-Op lymphopenia or Grade 1 lymphopenia trended towards NAR <8 but did not reach significance. No other relationship between lymphopenia (including grade) following treatment and tumour response, pCR, NAR score was observed. Between the post-CRT and pre-op time point most patients had a normal lymphocyte count or had mild lymphopenia (n=89 41%) This was followed by patients moving from Grade 0/1 to Grade 2+ (n=71 32%), then by patients staying in Grade 2+ at both time points (n=49 (22) and finally by patients moving from Grade 2+ to Grade 0/1 (n=10 5%). There was however no association between these groups and any measure of treatment response.

Table 3.4 Relationship between lymphopenia and treatment response measured by tumour regression grade, pathological complete response and NAR score (NAR<8 good prognosis; NAR _>8 intermediate / poor prognosis). Numbers in parenthesis indicate percentages. P values for chi square testing.

		Tumour Response			pCR			NAR		
		Good (TRG 0/1)	Poor (TRG 2/3)	P value	Yes	No	P value	<8	_>8	P value
Post-CRT lymphocyte count	Low	60 (78)	110 (83)	0.467	31 (71)	148 (84)	0.101	13 (26)	28 (16)	0.270
	Normal	17 (22)	22 (6)		13 (29)	28 (16)		37 (74)	142 (83)	
	High	0	1 (1)		0	1 (1)		0	1 (1)	
Post-CRT lymphopenia	No	17 (22)	23 (17)	0.609	13 (30)	29 (16)	0.061	13 (26)	29 (17)	0.152
	G1	20 (26)	34 (26)		9 (21)	49 (28)		11 (22)	47 (28)	
	G2	25 (33)	48 (36)		15 (34)	61 (35)		18 (36)	58 (34)	
	G3	14 (18)	28 (21)		6 (14)	38 (22)		7 (14)	37 (22)	
	G4	1 (1)	0		1 (2)	0		1 (2)	0	
Post-CRT lymphopenia	No / G1	37 (48)	57 (43)	0.455	22 (5)	78 (44)	0.479	24 (48)	76 (44)	0.657
	G2+	40 (52)	76 (57)		22 (50)	99 (56)		26 (52)	95 (56)	
	Low	48 (59)	84 (63)	0.628	25 (56)	113 (62)	0.593	29 (56)	109 (63)	0.553
	Normal	33 (41)	49 (37)		20 (44)	67 (37)		23 (44)	64 (37)	

Pre-OP lymphocyte count	High	0	1 (1)		0	1		0	1	
Pre-OP lymphopenia	No	33 (41)	50 (37)	0.858	20 (44)	68 (38)	0.635	23 (44)	65 (37)	0.383
	G1	28 (35)	49 (36)		16 (36)	62 (34)		20 (39)	58 (33)	
	G2	18 (22)	29 (22)		7 (16)	44 (24)		8 (15)	43 (25)	
	G3	2 (2)	6 (4)		2 (4)	7 (4)		1 (2)	8 (5)	
Pre-Op lymphopenia	No / G1	61 (75)	99 (74)	0.816	36 (80)	130 (72)	0.266	43 (83)	123 (71)	0.085
	G2+	20 (25)	35 (26)		9 (20)	51 (28)		9 (17)	51 (29)	
Lymphopenia change from Post-CRT to Pre-Op	G0/1 static	32 (42)	52 (40)	0.564	20 (46)	69 (39)	0.708	22 (44)	22 (44)	0.411
	G0/1 to G2+	26 (34)	44 (34)		15 (24)	56 (32)		52 (31)	19 (38)	
	G2+ to G0/1	5 (7)	4 (3)		2 (4)	8 (5)		8 (5)	2 (4)	
	G2+ static	14 (18)	31 (24)		7 (16)	42 (24)		42 (25)	7 (14)	

3.4 Discussion

This study demonstrates the majority of patients develop lymphopenia during neoadjuvant treatment which reduces by the preoperative time point. The severity / grade of lymphopenia (G2+) peaks post-CRT at 55% but reduces to 26% pre-operatively. This is in keeping with earlier literature in rectal cancer. [186]

There was a trend towards post-CRT lymphopenia being associated with mild anaemia and the absence of lymphopenia trending towards the absence of anaemia, but this did not reach significance. There was no clear association between post-CRT lymphopaenia, clinicopathological factors nor markers of systemic inflammation. However, pre-operative lymphopenia (G2+) was associated with an elevated CRP and mGPS. The absence of pre-operative lymphopenia was associated with a normal mGPS. Our study did not demonstrate a clear relationship between lymphopenia and treatment response.

The relationship between radiation and lymphopenia is considered to be multifactorial with a subset of patients having a persistent chronic lymphopenia which lasts for years following treatment. A potential hypothesis is these patients lose circulating lymphocytes and those in reservoir organs (particularly bone marrow), and lack the homeostatic rise of cytokines IL-7 and IL-15 required for clonal expansion. [185]

In our study, there was only 1 patient with grade 4 lymphopaenia. Grades 2 and 3 were grouped together as only 20% had grade 3 lymphopenia following CRT, and 4% preoperatively. It is possible an association with treatment response was not observed here due to weakening the signal with this combination. Heo et al observed a sustained lymphocyte ratio of ≥ 0.35 at 4 weeks was associated with pCR. [242] It is possible that we did not observe the same results as we assessed the lymphopenia over longer intervals of time (post-CRT included values from last week of radiotherapy to 4 weeks after radiotherapy and Pre-Op from 12-20 weeks after commencing radiotherapy). A further

limitation to our study was assessment of total lymphocyte count from peripheral blood and not obtaining the differential lymphocyte count. Tada et al have previously shown patients with a higher pre-CRT T lymphocyte, helper T lymphocytes and B lymphocyte counts were associated with improved response to chemoradiotherapy. Reduction in these counts were observed with chemoradiotherapy, but post-CRT levels of lymphocyte types were not associated with treatment response. Liu et al have shown a high absolute lymphocyte count nadir following CRT has shown to be associated with improved response. This was also associated with increased CD4+ helper T Cell, CD8+ cytotoxic T cell and CD68+ macrophage counts with resection specimens. [223]

Additionally, tumour infiltrating lymphocytes (cytotoxic CD8 + and regulatory CD4+ TILs) have a major role in tumour control within the tumour microenvironment. The relationship between circulating lymphocyte counts and tumour infiltering lymphocytes is complex and not well understood. Sawada et al did not identify any correlation between CD8+ TIL density and peripheral lymphocyte counts. [214] Therapeutic interventions such as T cell based immune checkpoint inhibitors aim to target negative co-stimulation receptors such as CTLA4, PD-1 and PD-L1 to prevent T cell exhaustion and tumour cells attenuating T cell activation. [243]

In summary despite a significant proportion of patients developing lymphopenia following chemoradiotherapy, this was not associated with treatment response. Following on from this I believe the way to understand these changes in depth was to have a standardised sampling protocol for patients undergoing neoadjuvant therapy assessing the markers of systemic inflammation already discussed in this and previous chapters but also combining this with tumour sampling to better understand the relationship between the local tumour microenvironment, systemic inflammation and treatment response. This is assessed in the Pilot Study in Chapter 6. The next chapter further investigates the association between anaemia and treatment response developing from the observations presented in chapter 2.

4 An investigation into the influence of anaemia on response to neoadjuvant chemoradiotherapy

4.1 Introduction

The antitumour activity induced by radiation is believed to be via the production of oxygen producing free radicals. Hypoxia reduces the availability of these free radicals and thus reduces radiation induced DNA damage. Anaemia reduces the oxygen carrying capacity of blood and liked to intratumoural hypoxia. The relationship between tissue hypoxia and resulting radioresistance is well documented in solid tumours of the head, neck and cervix. [244] [245]. Tumour hypoxia also results in genomic instability and mutations which result in neoplastic progression and angiogenesis which results in more aggressive tumours and distant metastases. [246] [247]

Walter and colleagues found a third of patients undergoing neoadjuvant CRT were anaemic at baseline and experienced a decline in haemoglobin during treatment. Pre-treatment anaemia was less likely to be associated with improved tumour regression. [248] This group further demonstrated a mild level of anaemia (Hb<12g/dL) was associated with tumour regression and mortality rates. [249]

Lee and colleagues found baseline anaemia was associated with more advanced clinical T stage, clinical nodal presence and lower rates of sphincter preserving surgery. Khan and colleagues described baseline haemoglobin being inversely related to the length of rectal cancer and clinical T stage but not related to clinical N stage, distance to anal verge or baseline CEA. Both groups found anaemia was associated with lower rates of pathological complete response. Despite anaemia being associated with increase local recurrence Lee et

al did not find this affected overall or disease specific survival [250] On the contrary other groups found anaemia was associated with poorer overall survival. [251] [252, 253]

Bong et al demonstrated a higher rate of poor response and non-complete response in in the presence of anaemia (Hb<9 g/dL) as well as those who underwent transfusion for anaemia before or during neoadjuvant chemoradiotherapy for rectal cancer. [254] However Rodrigues et al found anaemia (<13gL for males and <12 for females) was associated with poorer cancer specific and overall survival, especially in those aged over 75 years. [255]

McGrane et al reported poor tumour regression and higher mortality rates in the presence of anaemia (Hb <12g/L). [249] Berardi and colleagues demonstrated that Hb >12g/dL was associated with improved tumour downstaging and improved disease free but not overall survival. It was not clear if anaemia was the cause of adverse prognosis or a marker of more advanced tumours [256] Anaemia (mild & severe) has previously been linked to tumour necrosis which was associated with poorer cancer specific survival. [257]

Our group has previously assessed the impact of anaemia on neoadjuvant chemotherapy in oesophagogastric cancers finding 34% patients were anaemic at baseline and 59% pre-operatively. Anaemia was associated with increased clinical N staging, higher mGPS, higher intraoperative transfusion and poorer overall survival. [254] McSorley et al has previously described the association between normocytic anaemia, systemic inflammation and poorer survival in patients with colorectal cancer undergoing surgery. [258]

The literature has concentrated anaemia at baseline or pre-op anaemia and its relationship with treatment response. Little is known about whether development of anaemia during or after pelvic chemoradiation is associated with treatment response. Nor is it clear why anaemia may develop to a greater extent in some patients. In this chapter I aimed to investigate the presence of anaemia and development of anaemia at the three time points (baseline / post-CRT / pre-op). Specifically, I aim to identify any association between the

development of anaemia and treatment response, allowing its utilisation as a biomarker of response. My hypothesis is baseline anaemia or the development of anaemia during neoadjuvant therapy is associated with poorer treatment response.

4.2 Methods

The same patient database described earlier (in chapter 2), the systemic inflammatory response chapter, was used to study the effect of anaemia on treatment response. All patients had biopsy proven adenocarcinoma of the rectum with pre-treatment MRI and CT imaging to assess clinical TNM stage. Radiotherapy dose was usually 45Gy in 25 fractions over 5 weeks with 3 patients receiving a boost to regional nodes (range 32-54Gy). Concomitant chemotherapy was oral capecitabine, intravenous 5-Flurourcail or capecitabine in combination with irinotecan or oxaliplatin.

Routine bloods tests taken in the 1-3 weeks before commencing CRT were defined as baseline bloods. Blood tests taken from the last week radiotherapy to 4 weeks after completing CRT were defined as post-treatment bloods. Blood tests taken 12-20 weeks after commencing CRT and within 2 weeks of surgery were defined as pre-operative bloods. All lab results were grouped according to standard thresholds of normal as per the local laboratory values. Anaemia in was defined as <130g/dL in men and <115g/dL as per the local laboratory values. If was further categorised as per National Cancer Institute Common Terminology Criteria for Adverse Event (CTCAE) version 5. This is the common terminology descriptors for adverse events related to medical treatments or procedures, in this case CRT. Grading is based on severity: grade 1 / mild low normal to 100 g/dL; grade 2 / moderate 100 – 80 g/dL; grade 3 / severe 80 – 65 g/dL; grade 4 / life threatening <65 g/dL. [259] m(GPS) and NAR score were calculated as previously described. MRI T or N downstaging was defined as a reduction in T or N stage on post

treatment imaging compared to pre-treatment assessment. Pathological tumour response from chemoradiotherapy was as previously described. Tumour response was dichotomised into good / near complete response (TRG 0/1) and incomplete / poor response (TRG 2/3).

Statistical analysis was undertaken with using SPSS (version 25.0; IBM SPSS Statistics, IBM Corp, Armonk, NY, USA). Variables were grouped using standard clinical thresholds or cut offs which have been established in literature. Comparison between groups was performed with the Chi square test, and Fisher's exact test was used where n was less than 5.

4.3 Results

Table 4.1 demonstrates 77% of patients were not anaemic at baseline but this fell to 61% post-CRT returned to 76% pre-operatively. The proportion of patients with mild anaemia increased from 20% at baseline to 32% post-CRT before returning to 22% pre-operatively. Moderate anaemia also increased from a baseline 3% to 7% post-CRT and returned to 2% pre-operatively. Median time from pre-CRT to post-CRT was 5 weeks, post-CRT to pre-op was 11 weeks and pre-CRT to pre-op was 17 weeks.

Table 4.1 Presence and grade of anaemia prior to and following CRT. P values for Chi squared testing.

Anaemia grade	Pre CRT	Post CRT	Pre Op	
No Anaemia (Grade 0)	177 (77)	136 (61)	170 (76)	<0.001
Mild Anaemia (Grade 1)	45 (20)	70 (32)	50 (22)	
Moderate Anaemia (Grade 2)	6 (3)	15 (7)	5 (2)	

CRT Chemoradiotherapy; No Anaemia >130g/dL men / >115g/dL women; Mild Anaemia 100-130g/dL men / 100-115g/dL women; Moderate Anaemia 100 – 80 g/dL

Table 4.2 demonstrates the relationship between the grade of anaemia during therapy and clinic-pathological factors. There was no relationship between age, gender, BMI and grade of anaemia. Clinical T4 tumours were associated with moderate anaemia following therapy but this was not observed at other time points. At the pre-operative stage moderate anaemia was associated with clinical node negative status (80% vs 25% p=0.011) and stage II disease (90% vs 23% p=0.034 which was not present at previous times. EMVI on MRI was more trended towards mild anaemia before and after therapy but did reach significance preoperatively. Before therapy and pre-operatively moderate anaemia was more common in those with no EMVI on MRI. Moderate anaemia was more common in low rectal cancers, but this was not significant. Smaller tumours were least associated with moderate anaemia before and after therapy. There were no patients with moderate pre-operative anaemia who had ypT0-2 cancers. There was no relationship between anaemia and pathological nodal staging. There was no relationship between grade of anaemia and resection margin nor tumour differentiation. The relationship between pathological EMVI and anaemia was not clinically clear with more patients with moderate anaemia before therapy or pre-operatively being associated with pathological EMVI. There was no relationship between the type of neoadjuvant chemotherapy used and anaemia.

Table 4.2. Relationship between clinic-pathological factors and the grade of anaemia. P values from Chi squared testing.

		Pre CRT			p	Post CRT			p	Pre Op			p
		No	Mild	Moderate		No	Mild	Moderate		No	Mild	Moderate	
Age (years)	<55	33 (19)	10 (22)	1 (17)	0.982	27 (20)	15 (21)	1 (7)	0.582	33 (19)	7 (14)	1 (20)	0.711
	55 – 74	112 (63)	28 (62)	4 (67)		87 (64)	40 (57)	11 (73)		110 (65)	31 (62)	3 (60)	
	≥75	32 (18)	7 (16)	1 (17)		22 (16)	15 (21)	3 (20)		27 (16)	12 (24)	1 (20)	
Sex	Female	68 (38)	18 (40)	2 (33)	0.947	56 (41)	22 (31)	8 (53)	0.196	71 (42)	14 (28)	2 (40)	0.213
	Male	109 (62)	27 (60)	4 (67)		80 (59)	48 (69)	7 (47)		99 (58)	36 (72)	3 (60)	
BMI	<25	50 (36)	20 (56)	0	0.046	37 (36)	25 (46)	7 (54)	0.186	52 (39)	16 (41)	1 (50)	0.646
	25-30	49 (36)	10 (28)	4 (80)		37 (36)	17 (31)	6 (46)		45 (33)	16 (41)	1 (50)	
	>30	39 (29)	6 (17)	1 (20)		30 (28)	13 (19)	0		38 (28)	7 (18)	0	
MRI	T2	14 (8)	1 (2)	0	0.154	12 (9)	3 (4)	0	0.027	10 (6)	2 (4)	1 (20)	0.385

	T3	137 (77)	31 (69)	5 (83)		102 (75)	56 (80)	8 (53)		134 (79)	36 (72)	3 (60)	
	T4	26 (15)	13 (29)	1 (17)		22 (16)	11 (16)	7 (47)		26 (15)	12 (24)	1 (20)	
MRI	N0	48 (27)	13 (29)	3 (50)	0.467	40 (29)	16 (23)	6 (40)	0.346	42 (25)	18 (36)	4 (80)	0.011
	N1/N2	128 (73)	32 (71)	3 (50)		96 (71)	54 (77)	9 (60)		128 (75)	32 (64)	1 (20)	
Tumour Height from anal verge (cm)	<5 (low)	76 (44)	13 (33)	4 (67)	0.142	58 (44)	22 (34)	9 (64)	0.287	64 (39)	23 (50)	4 (80)	0.305
	5 – 10 (mid)	65 (38)	14 (35)	2 (33)		50 (38)	26 (41)	3 (21)		64 (39)	15 (33)	1 (20)	
	>10 (upper)	30 (17)	13 (33)	0		24 (18)	16 (25)	2 (14)		35 (22)	8 (17)	0	
Tumour Size	<4	28 (16)	7 (15)	0	0.024	24 (18)	9 (13)	2 (13)	0.018	24 (15)	9 (19)	1 (20)	0.438
	4-7	132 (77)	26 (61)	5 (83)		102(77)	46 (69)	9 (60)		126 (76)	31 (65)	4 (80)	
	≥8	12 (7)	10 (23)	1 (17)		7 (5)	12 (18)	4 (27)		15 (9)	8 (17)	0	
Clinical TNM Stage	I	4 (2)	1 (2)	0	0.737	4 (3)	1 (1)	0	0.534	3 (2)	2 (4)	0	0.034
	II	44 (25)	12 (27)	3 (50)		36 (27)	15 (21)	6 (40)		39 (23)	16 (32)	4 (80)	

	III	129 (73)	32 (71)	3 (50)		96 (71)	54 (77)	9 (60)		128 (75)	32 (64)	1 (20)	
MRI EMVI	No	33 (42)	3 (16)	3 (60)	0.064	26 (45)	9 (25)	3 (50)	0.129	30 (42)	4 (16)	3 (75)	0.019
	Yes	46 (58)	16 (84)	2 (40)		32 (55)	27 (75)	3 (50)		42 (58)	21 (84)	1 (25)	
MRI Response MRI Regression Grade	MR TRG 0/1	51 (44)	16 (53)	0	0.192	38 (41)	22 (49)	6 (86)	0.178	50 (46)	13 (42)	2 (50)	0.645
	MR TRG 2	54 (47)	14 (47)	0		45 (48.9)	22 (47)	1 (14)		52 (48)	15 (48)	1 (25)	
	MR TRG 3	11 (10)	0	0		9 (9)	2 (4)	0		7 (6)	3 (10)	1 (25)	
Pathological T staging	ypT0	41 (23)	4 (9)	0	0.010	33 (24)	9 (13)	2 (3)	0.097	38 (22)	7 (14)	0	0.679
	ypT1	12 (7)	0	0		8 (6)	3 (4)	0		10 (6)	2 (4)	0	
	ypT2	35 (20)	5 (11)	0		25 (18)	13 (19)	0		30 (17)	8 (16)	1 (20)	
	ypT3	78 (45)	31 (69)	6 (100)		64 (47)	37 (53)	12 (80)		83 (49)	29 (58)	3 (60)	
	ypT4	10 (6)	5 (11)	0		6 (4)	8 (11)	1 (7)		9 (5)	4 (8)	1 (20)	
Pathological N staging	ypN0	129 (73)	29 (64)	5 (83)	0.660	101 (74)	47 (67)	10 (67)	0.627	119 (70)	40 (80)	3 (60)	0.497
	ypN1	37 (21)	11 (24)	1 (16)		29 (21)	14 (20)	4 (27)		39 (23)	7 (14)	1 (20)	

	ypN2	11 (6)	5 (11)	0		6 (4)	9 (13)	1 (7)		12 (7)	3 (6)	1 (20)	
Resection Margin	R0	158 (89)	38 (84)	5 (83)	0.626	121 (89)	61 (87)	12 (80)	0.591	152 (89)	42 (84)	4 (80)	0.501
	R1 (≤1mm to CRM)	19 (11)	7 (16)	1 (17)		15 (11)	9 (13)	3 (20)		18 (11)	8 (16)	1 (20)	
EMVI	No	33 (42)	3 (15)	3 (60)	0.064	26 (45)	9 (25)	3 (50)	0.129	30 (42)	4 (16)	3 (75)	0.019
	Yes	46 (58)	16 (84)	2 (40)		32 (55)	27 (75)	3 (50)		42 (58)	21 (84)	1 (25)	
Tumour differentiation	Well, / Moderate	146 (91)	41 (95)	3 (75)	0.320	112 (93)	61 (90)	11 (100)	0.474	140 (92)	42 (91)	5 (100)	0.791
	Poor	14 (9)	2 (5)	1 (25)		9 (7)	7 (10)	0		13 (8)	4 (9)	0	
Chemotherapy	Capecitabine	116 (88)	31 (86)	3 (75)	0.712	87 (87)	48 (89)	10 (83)	0.651	112 (90)	32 (78)	4 (100)	0.358
	5-FU	13 (10)	5 (14)	1 (25)		10 (10)	6 (11)	2 (17)		11 (9)	8 (20)	0	
	Other *	3 (2)	0	0		3 (3)	0	0		2 (1)	1 (2)	0	

No Anaemia >130g/dL men / >115g/dL women; Mild Anaemia 100-130g/dL men / 100-115g/dL women; Moderate Anaemia 100 – 80 g/dL

BMI body mass index; TNM American Joint Committee Cancer (AJCC) TNM system; EMVI extramural vascular invasion; RT radiotherapy; CRT chemoradiotherapy; 5-FU Fluorouracil; * chemotherapy other: 1 Capecitabine & Irinotecan (Aristotle Study Intervention arm); 2 Xelox (Oxaliplatin & Capecitabine)

Table 4.3 Relationship between grade of serum markers of the systemic inflammatory response and anaemia. P values indicate chi square testing.

		Pre CRT			p	Post CRT			p	Pre Op			p
		No	Mild	Moderate		No	Mild	Moderate		No	Mild	Moderate	
White cell count (10^9L)	<4	4 (2)	1 (2)	0	0.637	21 (15)	21 (30)	3 (20)	0.179	20 (12)	6 (12)	2 (40)	0.341
	4-11	157 (89)	37 (82)	6 (100)		113 (83)	48 (69)	12 (80)		147 (86)	44 (88)	3 (60)	
	>11	16 (9)	7 (16)	0		2 (2)	1 (1)	0		3 (2)	0	0	
NLR	<3	113 (64)	18 (40)	2 (33)	0.007	24 (18)	11 (16)	1 (7)	0.544	53 (31)	18 (36)	2 (40)	0.762
	≥3	64 (36)	27 (60)	4 (67)		112 (82)	59 (84)	14 (93)		117 (69)	32 (64)	3 (60)	
Albumin (g/L)	<35	25 (14)	18 (40)	4 (67)	<0.001	26 (19)	30 (43)	13 (87)	<0.001	27 (16)	21 (44)	2 (40)	<0.001
	≥35	151 (86)	27 (60)	2 (33)		108(81)	39 (67)	2 (13)		138 (84)	27 (56)	3 (60)	
CRP (mg/L)	≤10	80 (87)	24 (80)	2 (40)	0.019	88 (81)	44 (73)	4 (31)	<0.001	88 (90)	27 (71)	3 (100)	0.018
	>10	12 (13)	6 (20)	3 (60)		21 (19)	16 (27)	9 (69)		10 (10)	11 (29)	0	

TRL	No	38 (22)	4 (9)	0	0.207	32 (23)	8 (11)	2 (13)	0.058	66 (39)	19 (38)	2 (40)	0.885
	Grade 1	44 (26)	12 (27)	2 (33)		39 (29)	14 (20)	5 (33)		61 (36)	16 (32)	1 (20)	
	Grade 2 +	88 (52)	29 (64)	4 (67)		65 (48)	48 (69)	8 (53)		43 (25)	15 (30)	2 (40)	
mGPS	0	80 (87)	24 (80)	2 (40)	0.011	88 (81)	44 (73)	4 (31)	<0.001	88 (90)	27 (71)	3 (100)	0.072
	1	8 (9)	1 (3)	1 (20)		9 (8)	4 (7)	1 (8)		6 (6)	5 (13)	0	
	2	4 (4)	5 (17)	2 (40)		12 (11)	12 (20)	8 (61)		4 (4)	6 (16)	0	

No Anaemia >130g/dL men / >115g/dL women; Mild Anaemia 100-130g/dL men / 100-115g/dL women; Moderate Anaemia 100 – 80 g/dL

RT radiotherapy; WCC total white cell count; NLR neutrophil to lymphocyte ratio; CRP C-reactive protein; TRL treatment related lymphopenia; mGPS modified Glasgow prognostic score.

Table 4.3 demonstrates the relationship between markers of systemic inflammation and grade of anaemia at each time point. A larger proportion of patients with NLR ≥3 had mild or moderate baseline anaemia. This was not observed at post CRT or pre-operatively. There was an association between hypalbuminaemia, CRP, mGPS and grade of anaemia across all 3 time points. Treatment related lymphopenia (post-CRT) trended towards an association to the grade of post-CRT anaemia. There was no association between white cell count and grade of anaemia at any time point.

Table 4.4.1-4.4.3. undertakes univariate and multivariate analysis of previously demonstrated significant clinical characteristics, marker of systemic inflammation and the likelihood of anaemia at each time point. On univariate analysis larger tumour (≥8cm), more advanced pathological T stage (ypT3/4), hypoalbuminemia, post-CRT CRP >10 and post-CRT mGPS (2) were associated with baseline anaemia. Baseline CRP>10 and elevated mGPS (2) trended towards a positive association with anaemia but did not reach significance. Only tumour size, pathological T stage, baseline and post CRT hypoalbuminemia, and baseline mGPS were independently associated with baseline anaemia.

On univariate analysis larger tumour size, advanced pathological T stage, post-CRT and pre-op hypoalbuminemia, elevated CRP and elevated mGPS was associated with post-CRT anaemia. Only baseline hypoalbuminemia was independently associated with post-CRT anaemia.

There were no clinical characteristics associated with pre-operative anaemia. Pre-operative anaemia was associated with post CRT and pre-operative hypoalbuminemia, elevated CRP and an mGPS of 2. Only pre-operative hypoalbuminemia was independently associated with pre-operative anaemia. There was no association between elevated NLR and anaemia at any time point.

Table 4.4.1 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors, markers of systemic inflammation and pre-CRT anaemia. Odds ratio and 95% confidence interval in parenthesis.

		Pre-CRT Anaemia			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
BMI	<25	1			
	25-30	0.67(0.30-1.46)	0.311		
	>30	0.42(0.16-1.09)	0.074		
Tumour Size (cm)	<4,	1			
	4-7	0.98(0.39-1.43)	0.960	1.46(0.29-7.33)	0.643
	≥8	4.79(0.03-3.67)	0.029	5.21(0.76-35.86)	0.093
Clinical TNM	I	1			
	II	1.36(0.14-13.18)	0.789		
	III	1.13(0.12-10.38	0.917		
Pathological T stage	ypT0	1			
	ypT1	0	0.999	0	0.999
	ypT2	1.46(0.37-5.88)	0.591	3.44(0.38-30.84)	0.269
	ypT3	4.80(1.60-14.40)	0.005	9.34(1.77-48.28)	0.008
	ypT4	6.83(1.59-29.32)	0.010	3.45(0.29-40.81)	0.325
Pathological EMVI	No	1			
	Yes	1.26(0.63-2.53)	0.516		
Pre-CRT NLR	No	1			
	Yes	1.46(0.84-2.52)	0.176		
Post-CRT NLR	<3	1			
	≥3	1.93(0.61-2.77)	0.490		
Pre-Op NLR	<3	1			
	≥3	0.70(0.39-1.24)	0.217		
Pre-CRT Albumin (g/L)	≥35	1		1	
	<35	4.96(2.44-10.07)	<0.001	3.71(1.18-11.62)	0.025

Post-CRT Albumin (g/L)	≥35	1			
	<35	4.36(2.38-7.98)	<0.001	5.38(1.62-17.84)	0.006
Pre-Op Albumin (g/L)	≥35	1			
	<35	2.27(1.18-4.39)	0.014	0.66(0.14-3.02)	0.493
Pre-CRT CRP (mg/L)	<10	1			
	≥10	2.65(0.98-7.21)	0.056	1.75(0.44-6.96)	0.425
Post-CRT CRP (mg/L)	<10	1			
	≥10	2.18(1.11-4.30)	0.024	0.91(0.12-6.72)	0.927
Pre-Op CRP (mg/L)	<10	1			
	≥10	1.71(0.66-4.44)	0.273		
Pre-CRT mGPS	0	1			
	1	1.79(0.45-7.05)	0.408		
	2	3.81(0.95-15.21)	0.058	4.89 (0.65-37.03)	0.124
Post-CRT mGPS	0	1			
	1	1.02(0.32-3.21)	0.975		
	2	3.06(1.34-6.78)	0.006	0.61(0.07-5.06)	0.640
Pre-Op mGPS	0	1			
	1	1.68(0.48-5.81)	0.416		
	2	1.75(0.45-6.84)	0.424		

BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; CRP C reactive protein; mGPS modified Glasgow Prognostic Score.

Table 4.4.2 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors, markers of systemic inflammation and post-CRT anaemia. Odds ratio and 95% confidence interval in parenthesis.

		Post CRT Anaemia			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
BMI*	<25				
	25-30	0.72 (0.36-1.45)	0.357		
	>30	0.50(0.24-1.12)	0.092		
Tumour Size (cm)	<4,				
	4-7	1.18(0.54-2.59)	0.685		
	≥8	4.99 (1.60-15.58)	0.006	0.91(0.31-2.69)	0.862
Clinical TNM	I	1			
	II	2.33(0.24-22.28)	0.462		
	III	2.63(2.29-24.03)	0.393		
Pathological T stage	ypT0	1			
	ypT1	1.13(0.25-5.00)	0.877		
	ypT2	1.56(0.60-4.06)	0.362		
	ypT3	2.30(1.06-5.00)	0.036		
	ypT4	4.50(1.31-15.52)	0.017	0.93(0.58-1.51)	0.777
Pathological EMVI	No	1			
	Yes	1.07(0.57-2.02)	0.825		
Pre-CRT NLR	<3	1			
	≥3	1.48(0.80-2.73)	0.207		
Post-CRT NLR	<3	1			
	≥3	1.10(0.47-2.60)	0.827		
Pre-Op NLR	<3	1			
	≥3	0.79(0.42-1.50)	0.467		
Pre-CRT Albumin (g/L)	≥35	1			
	<35	1.67 (0.81-3.40)	0.157		

Post-CRT Albumin (g/L)	≥35	1			
	<35	3.72(1.95-7.08)	<0.001	1.50(-.46-4.97)	0.504
Pre-Op Albumin (g/L)	≥35	1			
	<35	3.92(1.98-7.75)	<0.001	0.89(0.19-4.18)	0.879
Pre-CRT CRP (mg/L)	<10	1			
	≥10	1.09(0.83-3.08)	0.877		
Post-CRT CRP (mg/L)	<10	1			
	≥10	3.84(1.94-8.02)	<0.001	1.36(0.16—11.49)	0.779
Pre-Op CRP (mg/L)	<10	1			
	≥10	3.22(1.25-8.36)	0.016	6.19(0.66-57.670	0.110
Pre-CRT mGPS	0	1			
	1	0.68(1.36-3.39)	0.637		
	2	1.55(0.42-5.71)	0.509		
Post-CRT mGPS	0	1			
	1	1.88(0.55-6.46)	0.314		
	2	5.18(2.26-11.88)	<0.001	0.61(0.03-13.63)	0.754
Pre-Op mGPS	0	1			
	1	2.44(0.70-8.59)	0.163		
	2	4.40(1.16-16.66)	0.029	4.12(0.41-42.63)	0.227

BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; CRP C reactive protein; mGPS modified Glasgow Prognostic Score.

Table 4.4.3 Univariate and Multivariate binary logistic regression assessing relationship between clinical factors, markers of systemic inflammation and pre-operative anaemia. Odds ratio and 95% confidence interval in parenthesis.

		Pre-Op Anaemia			
		Univariate Analysis		Multivariate Analysis	
		Hazard Ratio	P value	Hazard Ratio	P value
BMI	<25	1			
	25-30	0.72(0.53-2.53)	0.717		
	>30	0.56(0.21-1.49)	0.249		
Tumour Size (cm)	<4,	1			
	4-7	0.67(0.29-1.52)	0.337		
	≥8	1.23(0.41-4.00)	0.669		
Clinical TNM	I	1			
	II	0.77(0.12-4.98)	0.783		
	III	0.39(0.06-2.41)	0.309		
Pathological T stage	ypT0				
	ypT1	1.09(0.20-5.06)	0.925		
	ypT2	1.63(0.54-4.48)	0.384		
	ypT3	2.09(0.85-5.17)	0.109	1.01(0.31-3.23)	0.989
	ypT4	3.02(0.78-11.73)	0.111	1.39(0.14-14.05)	0.779
Pathological EMVI	No	1			
	Yes	0.88(0.43-1.81)	0.734		
Pre-CRT NLR	<3	1			
	≥3	1.48(0.80-2.73)	0.207		
Post-CRT NLR	<3	1			
	≥3	1.10 (0.57-2.60)	0.827		
Pre-Op NLR	<3	1			
	≥3	0.79(0.42-1.50)	0.476		
Pre-CRT Albumin (g/L)	≥35	1			
	<35	1.67(0.82-3.40)	0.157		

Post-CRT Albumin (g/L)	≥35	1			
	<35	2.59(1.28-4.25)	0.008	1.54(0.60-3.96)	0.367
Pre-Op Albumin (g/L)	≥35	1			
	<35	2.78(1.32-5.88)	0.007	4.50(1.83-11.04)	0.001
Pre-CRT CRP (mg/L)	<10				
	≥10	1.09(0.38-3.08)	0.877		
Post-CRT CRP (mg/L)	<10				
	≥10	3.84(1.84-8.02)	<0.001	1.06(0.25-4.53)	0.937
Pre-Op CRP (mg/L)	<10				
	≥10	3.23(1.25-8.36)	0.016	2.28(0.71-7.33)	0.166
Pre-CRT mGPS	0	1			
	1	0.68(0.14-3.39)	0.637		
	2	1.55(0.42-5.71)	0.509		
Post-CRT mGPS	0	1			
	1	1.88(0.55-6.47)	0.314		
	2	5.18(2.26-11.88)	<0.001	1.60(0.31-8.41)	0.577
Pre-Op mGPS	0	1			
	1	2.44(0.70-8.59)	0.163		
	2	4.40(1.16-16.66)	0.029	0.59(0.05-6.86)	0.673

BMI body mass index; EMVI extramural vascular invasion; CRT chemoradiotherapy; CEA carcinoembryonic antigen; CRP C reactive protein; mGPS modified Glasgow Prognostic Score.

Table 4.5 details the relationship between the anaemia during therapy and response measured by TRG, pCR and NAR score. Presence of anaemia at all three time points is associated with lower rates of good response (TRG2/3) 10% vs 31%, lower pCR 9% vs 26% and low NAR score (<8) 8 % vs 27% (p=0.003). With baseline moderate anaemia only 2% had a good response, while there were no pCRs or NAR <8 (p<0.05). Post-CRT anaemia was associated with lower rates of good response 26% vs 46% and lower pCR 23% vs 42% (p<0.05) but did not reach significance for low NAR 28 % vs 41% (p=0.084). With post-CRT moderate anaemia only 4% had a good response vs 8% having a poor response(p<0.05), 5% had pCRs vs 7% non-pCRs(p=0.056) and 4% had NAR <8 vs 7% NAR _>8 (p=0.213). Pre-operatively anaemia was associated with lower good response 14% vs 30%, lower pCR 13% vs 27% (p<0.05) and trended towards fewer low NAR score 15% vs 27% (p=0.083). Moderate anaemia pre-operatively was not associated with any cases of good response (p=0.015), pCR or low NAR score but did not reach significance for the latter measures of response (p=0.124 and p=0.160).

Table 4.5 Relationship between the grade of anaemia prior to and following CRT and treatment response. P values for Chi squared testing.

			Response (%)			pCR (%)			NAR score		
			Good (TRG 0/1)	Poor (TRG 2/3)	P value	Yes	No	P value	<8	_>8	P value
Pre CRT Anaemia	No	176 (77)	73 (90)	94 (69)	0.001	41 (91)	135 (74)	0.013	48 (92)	128 (73)	0.003
	Yes	52 (23)	8 (10)	42 (31)		4 (9)	48 (26)		4 (8)	48 (27)	
Pre CRT Anaemia	No	176 (77)	73 (90)	94 (69)	0.003	41 (91)	136 (74)	0.047	48 (92)	129 (73)	0.014
	Mild	45 (20)	7 (9)	36(27)		4 (9)	41 (22)		4 (8)	41 (23)	
	Moderate	6 (3)	1 (2)	5 (4)		0	6 (3)		0	6 (3)	
Post CRT Anaemia	No	136 (61)	57 (74)	72 (54)	0.004	34 (77)	102 (58)	0.017	36 (72)	100 (59)	0.084
	Yes	85 (38)	20 (26)	61 (46)		10 (23)	75 (42)		14 (28)	71 (41)	
Post CRT Anaemia	No	136 (62)	57 (74)	72 (54)	0.017	34 (77)	102 (58)	0.056	36 (72)	100 (59)	0.213
	Mild	70 (32)	17 (22)	51 (38)		8 (18)	62 (35)		12 (24)	58 (34)	
	Moderate	15 (7)	3 (4)	10 (8)		2 (5)	13 (7)		2 (4)	13 (7)	
Pre Op Anaemia	No	170 (76)	70 (86)	93 (70)	0.006	39 (87)	131 (73)	0.052	44 (85)	126 (73)	0.083
	Yes	55 (24)	11 (14)	40 (30)		6 (13)	49 (27)		8 (15)	47 (27)	
Pre Op Anaemia	No	170 (76)	70 (86)	93 (70)	0.015	39 (87)	131 (73)	0.124	44 (85)	126 (73)	0.160
	Mild	60 (22)	11 (14)	36 (27)		6 (13)	44 (24)		8 (15)	42 (24)	
	Moderate	5 (2)	0	4 (3)		0	5 (3)		0	5 (3)	

TRG tumour regression grade; pCR pathological complete response; NAR neoadjuvant rectal score

No Anaemia >130g/dL men / >115g/dL women; Mild Anaemia 100-130g/dL men / 100-115g/dL women; Moderate Anaemia 100 – 80 g/dL

Table 4.6 demonstrates the development of anaemia during therapy and response. Most patients did not develop anaemia during therapy. 17-18% patients developed anaemia immediately post-CRT, but this was not associated with tumour regression grade. When comparing baseline to pre-operatively, 10-11% patients developed anaemia. Patients who were anaemic from baseline to post-CRT or baseline to pre-operatively were more likely to have a poorer response 29% vs 8% and 20% vs 3% respectively (<0.05). This was also observed for pathological complete response (pCR). Anaemia baseline to post-CRT resulted in 9% pCR vs 24% non-pCR ($p=0.037$). Anaemia from baseline to pre-operatively trended similarly 4% pCR vs 16% pCR but did not reach significance ($p=0.066$). The pattern of developing anaemia from baseline to post-CRT or from baseline to pre-operatively was associated with fewer number of patients with a low NAR score, 8% vs 25% and 4% vs 16% respectively ($p<0.05$).

Table 4.6 Relationship between treatment response (measured TRG, pCR and NAR) and change in the presence of anaemia from baseline to post-CRT or baseline to pre-operatively. P values indicate chi square testing.

		Response (%)		P value	pCR (%)		P value	NAR (%)		P value
		Good (TRG 0/1)	Poor (TRG 2/3)		Yes	No		<8	_>8	
Anaemic Baseline to Post-CRT	No – No	55 (71)	69 (52)	0.003	34 (77)	97 (55)	0.037	36 (72)	95 (56)	0.031
	No – Yes	14 (18)	22 (17)		6 (14)	32 (18)		10 (20)	28 (16)	
	Yes – Yes	6 (8)	39 (29)		4 (9)	43 (24)		4 (8)	43 (25)	
	Yes – No	2 (3)	3 (2)		0	5 (3)		0	5 (3)	
Anaemic Baseline to Pre-Op	No – No	64 (79)	79 (59)	0.002	37 (82)	112 (62)	0.066	42 (81)	107 (62)	0.030
	No – Yes	9 (11)	13 (10)		4 (9)	21 (12)		6 (12)	19 (11)	
	Yes – Yes	2 (3)	27 (20)		2 (4)	28 (16)		2 (4)	28 (16)	
	Yes – No	6 (7)	14 (11)		2 (4)	19 (11)		2 (4)	19 (11)	

TRG tumour regression grade; pCR pathological complete response; NAR neoadjuvant rectal score

4.4 Discussion

This chapter has demonstrated anaemia can be used as biomarker of poor treatment response. Baseline anaemia was associated with higher T stage cancers as previously described but there was no association with nodal staging. [250] [256] Baseline anaemia was associated with lower rates of good response, pCR and low NAR. No patients with baseline moderate anaemia had pCR or NAR <8. Post-CRT and pre-op anaemia was associated with fewer rates of good response and pCR but did not reach significance for low NAR. There were no good responses in patients with moderate anaemia preoperatively. [249] [254] This is reflective of poor tumour response in the presence of anaemia in the literature. To the best our knowledge this is the first study to find patients who had sustained anaemia from baseline to post-CRT had lower rates of good tumour response, pCR and NAR<8. Patients who had sustained anaemia from baseline to pre-op had lower rates of good tumour response, NAR<8 and trended toward lower rates of pCR.

The association between anaemia at baseline or during radiotherapy associated with poorer tumour response and non-pCR is in keeping with literature. Attempts to overcome this with perioperative blood transfusion have not been shown improve treatment response. [188]. Transfusion has been shown to increase postoperative inflammation, increase postoperative complications and result in poorer overall survival. [193] McSorley et al has previously described poorer overall survival in patients with colorectal cancer with normocytic anaemia compared to microcytic anaemia, likely as a result of systemic inflammation resulting in normocytic anaemia. [194] My study has again confirmed the independent association between baseline anaemia and an elevated mGPS. Only albumin remained

significant on multivariate analysis following chemoradiotherapy. Interestingly NLR was not associated anaemia. This highlights the difficulty in measuring systemic inflammation.

Pre-operative parenteral iron infusion was associated with the greater increase in haemoglobin in patients with operable colorectal cancers with iron deficiency anaemia compared to functional iron deficiency anaemia or anaemia of inflammation. All rectal cancers in the reference study were associated with iron deficiency anaemia unlike 23% in our cohort. [195] Anaemia of inflammation is caused by iron restriction from hepcidin-mediated hypoferremia and cytokine mediated suppression of erythropoiesis. Cytokine release causes increased hepcidin production in the liver, macrophage activation, increased red blood cell destruction, erythropoiesis suppression with the switch to leukopoiesis. [260]A Cochrane review of erythropoietin to target anaemia found no benefit to this therapy. [261]

The other main hypothesis of poorer response to radiotherapy in the context of anaemia is from hypoxia induced radioresistance. Invitro experiments have shown limited radiosensitivity when the partial pressure of oxygen falls below 25-30mmHg. Hypoxic tumours have a higher risk of being invasive, having metastatic potential and thus having poorer prognosis. [262-264]Potential future target therapies in pre-clinical studies include lipid A, its analogue OM-174, myeloid derived suppressor cell inhibitors and intravenous L-arginine, an isoform NO synthase inducer to modulate the L-arginine pathway. [265] [266] [267]

In conclusion our study mirrored previous studies which demonstrated anaemia was associated with a poor treatment response. In our study there were no patients with severe or life threatening anaemia. A limitation of our study was not collecting data on mean cell volume, transferrin or pre-operative transfusion. It is therefore not clear if the relationship

which has been observed is resultant from anaemia related hypoxia, from the immune effects caused by iron deficiency, or as anaemia being a marker of chronic inflammation or a combination of all.

Future work should address this to study if the type of anaemia influences response to neoadjuvant chemoradiotherapy, this is beyond the scope of this thesis.

The work in this thesis until now has relied on retrospectively collected blood results at varying timepoints rather than prospectively. The aim of the pilot study is to have a protocolised assessment of circulating makers of systemic inflammation long with tumour biopsies to study the local inflammatory response at clear time points.

5 An investigation of the immune priming effect of radiotherapy in rectal cancer - a prospective study protocol

5.1 Introduction

Previous chapters have assessed markers of systemic inflammation from serum blood sampling retrospectively. Due to the nature of these studies, there is a lack of standardisation of sampling timing and tests undertaken. All patients from the previously described chapters underwent long course chemoradiotherapy. We aimed to overcome these challenges with a prospective study using protocol specified specimen retrieval to assess local and systemic antitumour inflammatory changes occurring during neoadjuvant therapy following on from previously validated specimen sampling. [268] By better understanding the immune response from neoadjuvant therapy, this may allow for strategies to enhance the immune response using novel immune therapies thus enhancing treatment response. Currently immunotherapies are predominantly used in the context of metastatic disease or in trials.

The primary aim of this study was to assess patterns of immune response in patients undergoing short course radiotherapy and long course radiotherapy. Secondary aims were to assess: the peak of immune response; if tumour molecular make up effects immune response; types of immune response have a role in tumour response; patient acceptability of repeated endoscopic assessments with blood sampling. I am including this work within my thesis due to the considerable time and effort I had spent on the recruitment and processing of study patients, ethics amendments and working closely with lab staff at the Edwards lab for specimen processing.

5.2 Methods

This study underwent approval by the Research Ethics Committee and registered on the Health Research Authority database (IRAS ID 239609). The aim was to recruit 30 patients, 15 to short course radiotherapy and 15 to long course radiotherapy. Patients were identified through the colorectal cancer multidisciplinary team meeting and approached at Surgical or Oncology clinics at Glasgow Royal Infirmary. Each potential participants were given a Patient Information Sheet and at a follow up visit to provide informed consent in order to participate in the study. The study involved volunteering to allow additional targeted biopsies by proctosigmoidoscopy and blood samples to be taken for research purposes at 4 time points as they undergo treatment with radiotherapy for rectal cancer.

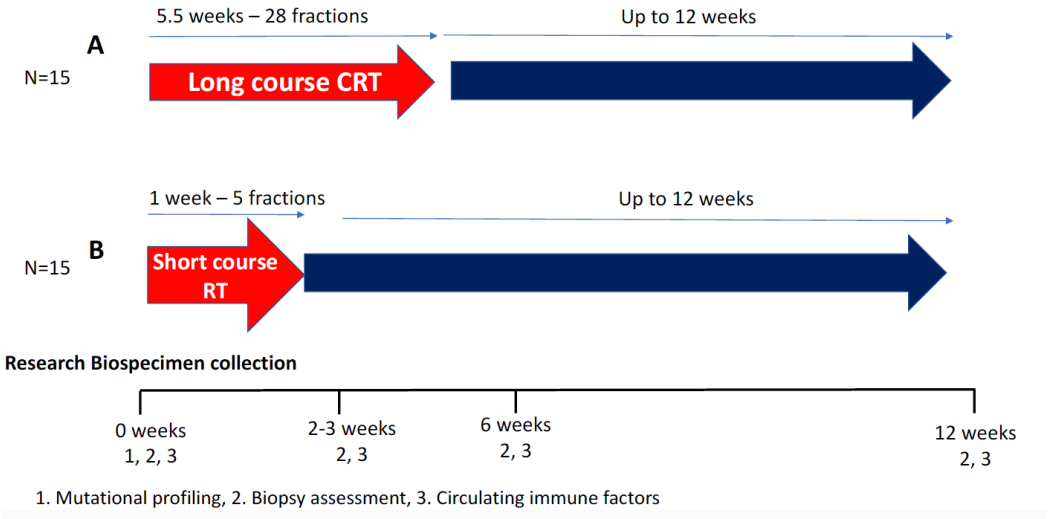
The Ethical approvals and funding were already in place during my involvement with the study. My role was identification and recruitment of patients to the study. I ensured blood and tissue sampling were undertaken as per protocol and transferred to Professor Edward's laboratory for further processing.

Inclusion criteria: Over 18 years of age; Patients with known rectal cancer (adenocarcinoma) which is due to be treated with radiotherapy; Capacity to provide informed consent; Willingness to allow additional tumour biopsies to be performed at clinical assessments; The ability to understand simple written and verbal English

Exclusion criteria: Under 18 years of age; Patients with bleeding disorders; Patients prescribed anticoagulants in whom a bleeding risk would be present (warfarin, dalteparin, apixaban); Patients with tumours at or below the dentate line of the anal canal

Patients underwent protocol specimen retrieval at 0, 3, 6 and 12 weeks in long course regimens, 0, 2, 6 and 12 weeks in short course regimens. Blood sampling was also performed at each timepoint (detailed in figure 6.1).

Figure 5.1. Biospecimen collection time points.



Biopsies were placed in formalin and transferred immediately to the Glasgow Biorepository, where they will be cold stored prior to tissue processing. Once tissue has been sectioned and approved by the pathologist for research use, the tissue was transferred to Professor Joanne Edwards lab at the Wolfson Wohl Cancer Research Centre, Glasgow. In the case of complete clinical response to radiotherapy, we would continue taking blood and biopsy samples when these patients are recalled for surveillance endoscopies by their treating clinical team. Tumour samples underwent molecular subtyping as baseline and then immunohistochemistry was performed at each biopsy time point to assess immune cell infiltrates (beyond the scope of this thesis). Blood sampling included routine differential blood count, C-reactive protein, albumin, and samples for cytokine profiling and flow cytometry (the latter beyond the scope of this thesis). Full study protocol and patient consent form attached as Appendix I and II.

5.3 Results

A total of 11 patients were recruited from November 2018 and December 2019. 3 patients did not manage to complete the study: 1 due to mortality from unrelated diverticular perforation; 1 who was had short course radiotherapy with palliative intent and could no longer manage additional hospital visits due to frailty; the last patient's specimen retrieval was abandoned after week 2 due to restrictions during the COVID pandemic. This prevented further recruitment to the study due to concerns for patient safety and social distancing restrictions. 3 of the patients were women with the remaining men. Table 1 details the baseline patient characteristics along with type of neoadjuvant therapy and outcome. 3 patients had metastatic disease at presentation. 6 patients underwent LCRT, and 5 patients had SCRT: 2 due to treatment with palliative intent; 2 who were undergoing systemic chemotherapy for metastatic disease; and 1 patient due to COVID restrictions. 1 patient had a complete clinical response. 3 patients underwent surgery of which 2 were TME resections and 1 was an anterior exenteration.

Table 5.1 Baseline clinical staging parameters, treatment and outcome.

Patient ID	Age	cT (MRI)	cN (MRI)	Tumour Size (cm)	Tumour height from AV (cm)	Clinical TNM staging	Metastasis at diagnosis	Therapy	Radiation Dose (Gy)	Chemotherapy	Outcome	Surgery
1	56	3	1	3.0	2.2	3	none	Long course	52	capecitabine	cCR	No surgery
2	57	3	2	4.7	3.8	3	none	Long course	52	capecitabine	Progression & death	No surgery
3	56	3	1	4.8	5.5	3	none	Long course	53	capecitabine	surgery	Anterior resection
4	56	3	2	6.3	6.0	3	none	Long course	47	5FU	surgery	Exenteration
5	80	4	2	7.0	2.5	4	liver	Short course	20	No chemotherapy	Progression & death	No surgery
6	36	3	0	3.2	2.7	2	none	Long course	52	capecitabine	surgery	AP Resection
7	54	4	0	3.0	3.2	2	none	Long course	52	capecitabine	progression	No surgery

8	39	4	2	4.5	15.0	4	liver	Short course & Chemo	25	FOLFIRI	progression	No surgery
9	84	4	1	10.0	9.0	3	none	Short course	25	No chemotherapy	symptom improvement (palliative)	No surgery
10	59	3	2	6.5	4.1	4	liver & lung	Short course & Chemo	25	XELOX	progression	No surgery
11	66	3	1	3.8		3	none	Short course	25	No chemotherapy	progression	No surgery

Figures 5.2. shows the graphical trends of blood sampling at the time intervals previously specified. Haemoglobin and white cell count fall in most patients. Fall in lymphocyte count is more pronounced than neutrophils with NLR rising in most patients. CEA initially falls in all patients but rises after week 2 in 2 patients who both had progressive metastatic disease. CRP fell for most patients until week 2, staying relatively static during treatment except for 3 patients for whom it remained persistently elevated for. This was reflected with an elevated mGPS for these patients. Albumin fell at week 2 but returned close to baseline by week 6.

Figures 5.3.1 and 5.3.2 shows patient 1 achieving a complete clinical response with endoscopic and MRI images from baseline and week 12. Figures 6.4.1 and 6.4.2 show patient 2 having evidence of disease progression from baseline to week 12. 2.

Figure 5.2.1 Haemoglobin over time

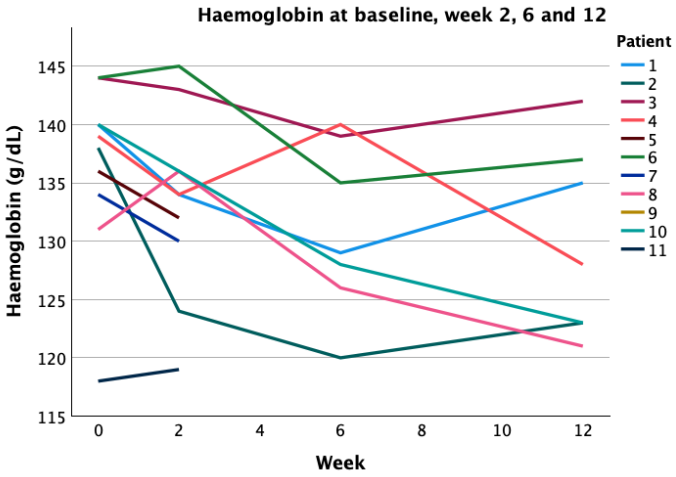


Figure 5.2.2 White cell count over time

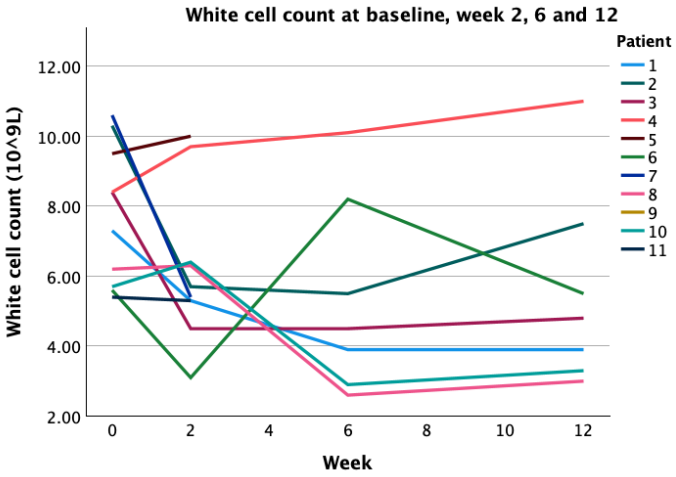


Figure 5.2.3 Neutrophil count over time

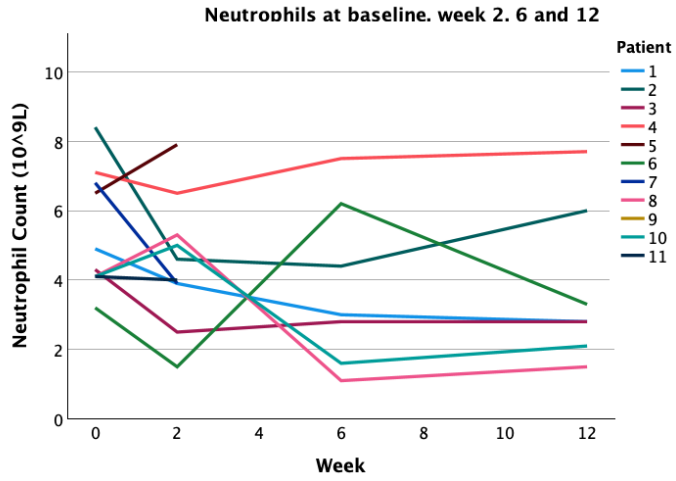


Figure 5.2.4 lymphocyte count over time

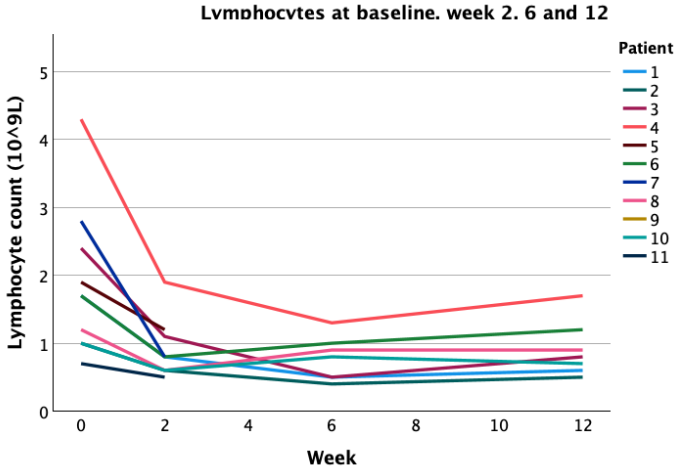


Figure 5.2.5 NLR over time

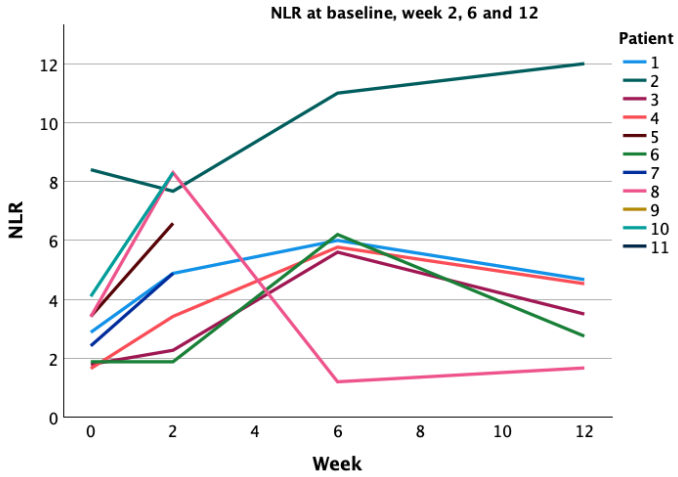


Figure 5.2.6 CEA over time

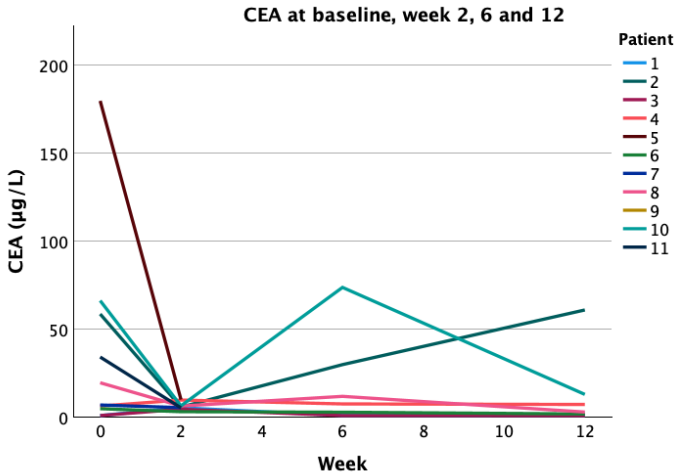


Figure 5.2.7 CRP over time

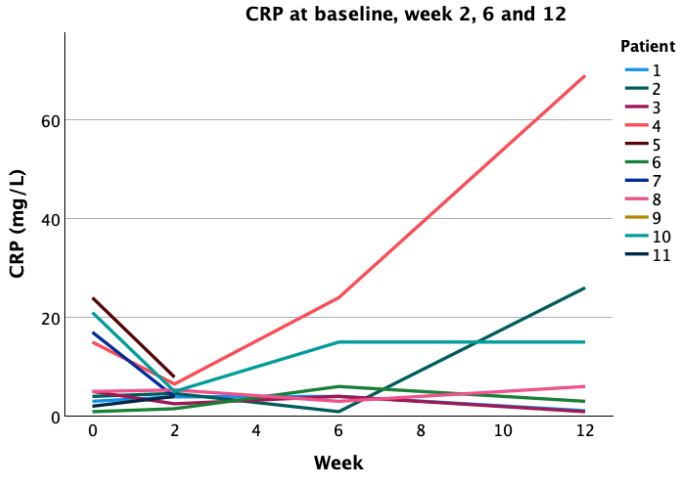


Figure 5.2.8 Albumin over time

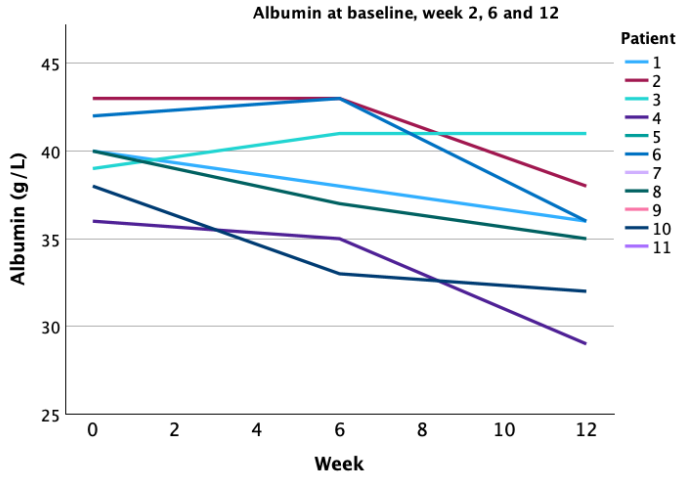


Figure 5.2.9 mGPS over time

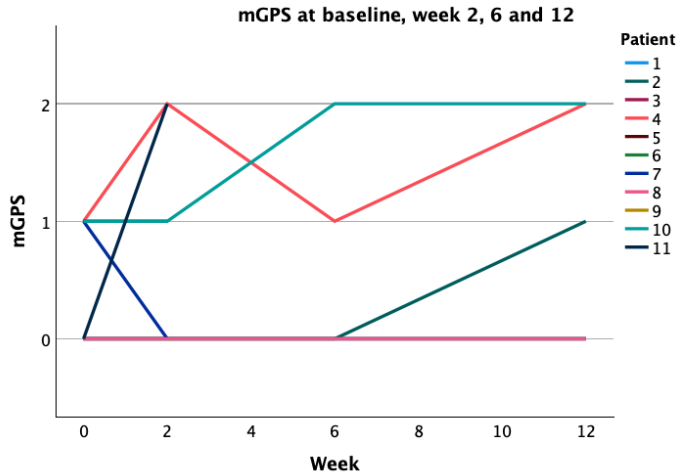


Figure 5.3.1. Baseline MRI pelvis and endoscopy with tumour indicated by white arrow

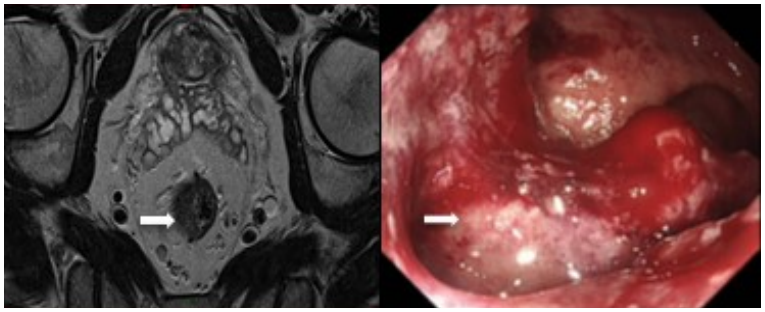


Figure 5.3.2. Week 12 MRI pelvis and endoscopy showing complete response. Scar indicated by black arrow

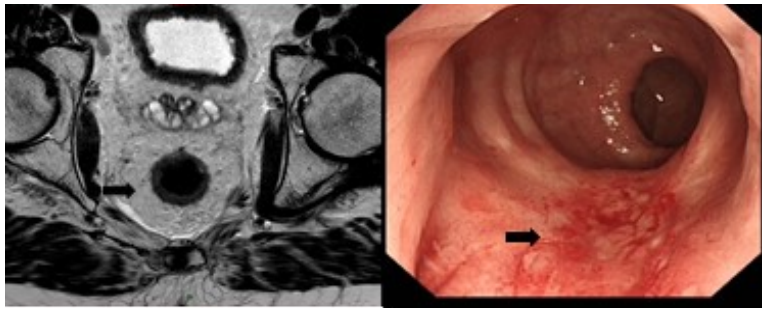


Figure 5.4.1. Baseline MRI pelvis and endoscopy with tumour indicated by white arrow

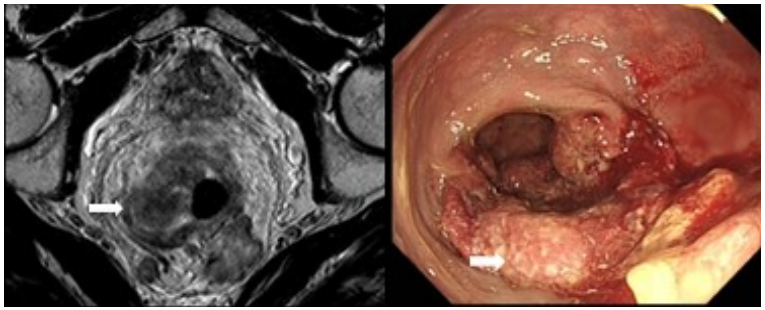
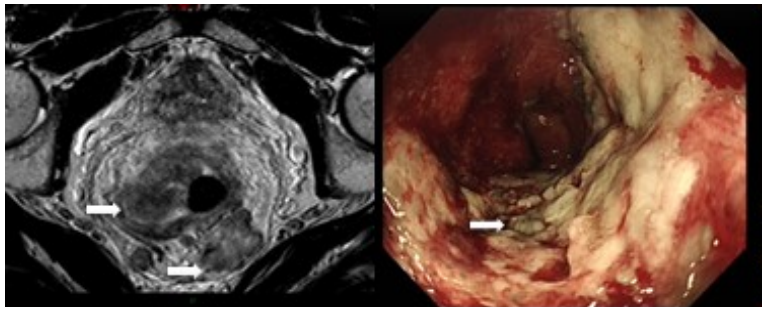


Figure 5.4.2. Week 12 MRI pelvis and endoscopy with significant residual disease. Tumour indicated by white arrow



5.4 Discussion

This pilot study achieved its goals of a serial protocolised specimen retrieval program of tissue and circulating markers during neoadjuvant therapy. 11 patients were recruited to the study before suspension due to the COVID pandemic. All patients managed to complete the retrieval program except 2, 1 due to frailty and 1 due to COVID and was well tolerated. There was nearly an even split of patients who underwent short course radiation with or without chemotherapy and long course chemoradiotherapy. 1 patient had a complete clinical response and 3 underwent surgery. Detailed statistical analysis was not undertaken due to the small numbers of patients. In addition to the circulating makers described additional samples were stored for future analysis of cytokine analysis and flow cytometry to assess circulating T cell and myeloid cell populations. Tumour samples were stored in formalin and fresh frozen for future analysis for immunohistochemistry assessment of: T cell response; myeloid response; antigen presentation; and immune checkpoint expression.

This would a comprehensive assessment of the local and systemic inflammatory response to neoadjuvant therapy. Preliminary tissue analysis using immune histochemistry, cytokine profiles and genomic analysis was undertaken which has fed into the larger ongoing study, but this is out with the scope of this thesis.

This proof of concept study serially assessing response of patients receiving standard of care treatment will run alongside the PRIME RT study (Clinicaltrials.gov NCT04621370). [269] PRIME RT has 2 arms:

1) Short course radiotherapy followed by FOLFOX chemotherapy with concurrent Durvalumab
2) Long course chemoradiotherapy followed by FOLFOX chemotherapy with concurrent Durvalumab

The study aims of assess if the addition of Durvalumab, an anti-PD1 immunotherapy agent, can improve rates of complete response to neoadjuvant therapy in rectal cancer and compare the long and short course radiotherapy strategies.

Our pilot study has shown demonstrated protocolised biospecimen retrieval during neoadjuvant therapy is feasible and tolerated by patients. It has allowed for setting up the infrastructure to run these studies. Biospecimens retrieved from this pilot study will allow in depth investigation of the mechanisms driving the systemic inflammatory response with cytokine profiling and in depth study of circulating subpopulations of white blood cells. Serial biopsies will allow assessment of the changing tumour microenvironment during therapy including that of patients who achieved complete clinical and pathological responses. This would also allow exploration of the mechanism linking the local and systemic inflammatory responses with the aim of identifying a reliable biomarker of treatment response.

6 Summary

The aim of this study was to comprehensively assess the relationship between markers of systemic inflammation and their relationship to response to chemoradiotherapy in rectal cancer.

Chapter 2 assessed the relationship between biomarkers of systemic inflammation at three time points (baseline, following neoadjuvant chemotherapy and preoperatively) and response to neoadjuvant chemotherapy. This chapter observed white cell counts, lymphocyte counts, haemoglobin and albumin decreased following neoadjuvant CRT and partially recovered afterwards. NLR increased during treatment whilst CEA decreased. Anaemia and high CEA were associated with poor treatment response. There was no relationship observed between NLR, CRP, mGPS and treatment response.

This chapter also assessed the relationship between changes in markers of systemic inflammation and its influence on response to CRT. NLR increased from baseline to following therapy and fell slightly preoperatively, predominantly from falling lymphocyte counts during chemoradiotherapy. CRP which remained low or fell during therapy was associated with improved NAR score. Despite the numbers of patients with an elevated mGPS rising during therapy neither CRP or mGPS changes were associated with tumour regression grade or pCR. Albumin which remained high following therapy was associated with improved tumour regression grade and pCR. Persistently elevated CEA was associated with lower rates of pCR and favourable NAR score.

Chapter 3 assessed the relationship between lymphopenia and its influence on response to CRT. The majority of patients developed mild / moderate lymphopenia following therapy, but lymphopenia was not associated with treatment response.

Chapter 4 assessed the relationship between anaemia and response to CRT. Baseline anaemia was associated with higher cancer T stage, an elevated mGPS and poor response to therapy with lower rates of pCR, tumour regression grade and lower numbers of patients with a low NAR score. Sustained anaemia during therapy was associated with lower rates of tumour response, pCR and low NAR score. Sustained anaemia from baseline to preoperatively was associated with lower rates of good tumour response and favourable NAR score.

Chapter 5 assessed the feasibility of a protocolised biospecimen retrieval of circulating markers of inflammation and tumour tissue. Despite the study having an even split of patients undergoing short and long course, the overall numbers in this study were limited. There are plans to undertake a detailed assessment of circulating markers of systemic inflammations and serial assessment of the tumour microenvironment during and after therapy which are out with the scope of this thesis.

7 Discussion

This thesis did not identify NLR, CRP and mGPS as biomarker of treatment response. In previous studies, the majority of studies have used cutoffs deemed by ROC analysis and log-rank testing. Having varying cutoffs make reproducibility more difficult. Previous studies have also concentrated on the survival rather than treatment response. Immune cell infiltration within the tumour microenvironment is associated with improved longer term oncological outcomes. The relationship between the local inflammatory response and systemic inflammatory response is not fully understood and there is no serum biomarker which reliably correlates with local immune cell tissue infiltration.

In our study anaemia was associated with poor response. Anaemia is associated with systemic inflammation. The mechanism of behind anaemia with systemic inflammation is not fully understood. It is difficult to know if poorer response is as a result of anaemia or systemic inflammation or both. Future study with serum iron profiles may understand if patients with iron deficiency anaemia have deferring treatment responses to patients with anaemia of chronic disease potentially identifying therapeutic options.

This thesis assessed the relationship between circulating markers of systemic inflammation and treatment response. With more time it would be beneficial to study the relationship between these serum marker and changes in the tumour microenvironment from baseline and following treatment in matched tissue specimens. Ongoing work from the pilot study will assess the relationship between local, systemic inflammatory response, particularly circulating cytokines and chemokines, and its relationship with treatment response.

Having a reliable biomarker of treatment response will allow greater precision in the management of rectal cancer. With the increasing use of total neoadjuvant therapy, increasing rates of complete response, and thus greater rates of organ preservation and

survival have been demonstrated in recent OPRA and RAPIDO studies. [270] [271] It is important to identify patients experiencing poorer response to conventional treatment as novel immunotherapy strategies may have a therapeutic role. Their role historically has been in the context of metastatic disease in immunogenic mismatch repair deficient / microsatellite stable tumours. Cereck et al have shown immunotherapy alone resulted in sustained complete clinical response in all patients with locally advanced microsatellite unstable cancers without significant toxicity allowing patients to avoid complications and side effects of chemoradiotherapy. [272] Low immunogenic profiles of microsatellite stable cancers are resistant to immune checkpoint blockade. Combination therapies have been attempted to convert a cold non-immunogenic cancer into a hot immunogenic cancer susceptible to immune checkpoint inhibition and improve response to chemoradiotherapy has varying success and the subject of ongoing clinical trials. It is therefore of vital importance a reliable biomarker can be identified to measure these responses to guide and target therapies for individual patients.

8 References

1. UK, C.R. *Bowel cancer incidence statistics*. 2022 01/07/2023]; Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer/incidence#heading-Four.
2. Allemani, C., et al., *Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries.* Lancet, 2018. **391**(10125): p. 1023-1075.
3. Stoffel, E.M. and C.C. Murphy, *Epidemiology and Mechanisms of the Increasing Incidence of Colon and Rectal Cancers in Young Adults.* Gastroenterology, 2020. **158**(2): p. 341-353.
4. Willaert, W., et al., *New insights into the surgical anatomy of the rectum: a review.* Acta Chir Belg, 2011. **111**(5): p. 261-72.
5. Jorge, J.M.N. and A. Habr-Gama, *Anatomy and Embryology of the Colon, Rectum, and Anus*, in *The ASCRS Textbook of Colon and Rectal Surgery*, B.G. Wolff, et al., Editors. 2007, Springer New York: New York, NY. p. 1-22.
6. Heald, R.J. and B.J. Moran, *Embryology and anatomy of the rectum.* Seminars in Surgical Oncology, 1998. **15**(2): p. 66-71.
7. Dekker, E., et al., *Colorectal cancer.* The Lancet, 2019. **394**(10207): p. 1467-1480.
8. Zampino, M.G., et al., *Rectal cancer.* Crit Rev Oncol Hematol, 2009. **70**(2): p. 160-82.
9. Bingham, S.A., et al., *Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study.* The Lancet, 2003. **361**(9368): p. 1496-1501.
10. Schatzkin, A., et al., *Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study234.* The American Journal of Clinical Nutrition, 2007. **85**(5): p. 1353-1360.
11. Ferrari, P., et al., *Lifetime and baseline alcohol intake and risk of colon and rectal cancers in the European prospective investigation into cancer and nutrition (EPIC).* International Journal of Cancer, 2007. **121**(9): p. 2065-2072.
12. Gonzalez, C.A. and E. Riboli, *Diet and Cancer Prevention: Where We Are, Where We Are Going.* Nutrition and Cancer, 2006. **56**(2): p. 225-231.
13. Ogimoto, I., A. Shibata, and K. Fukuda, *World Cancer Research fund/american institute of cancer research 1997 recommendations: Applicability to digestive tract cancer in Japan.* Cancer Causes & Control, 2000. **11**: p. 9-23.
14. Xi, Y. and P. Xu, *Global colorectal cancer burden in 2020 and projections to 2040.* Transl Oncol, 2021. **14**(10): p. 101174.
15. Stewart, B.W. and P. Kleihues, *World cancer report*. Vol. 57. 2003: IARC press Lyon.
16. Ahmed, R.L., et al., *The metabolic syndrome and risk of incident colorectal cancer.* Cancer: Interdisciplinary International Journal of the American Cancer Society, 2006. **107**(1): p. 28-36.
17. Karahalios, A., D.R. English, and J.A. Simpson, *Weight Change and Risk of Colorectal Cancer: A Systematic Review and Meta-Analysis.* American Journal of Epidemiology, 2015. **181**(11): p. 832-845.
18. McNabb, S., et al., *Meta-analysis of 16 studies of the association of alcohol with colorectal cancer.* Int J Cancer, 2020. **146**(3): p. 861-873.

19. Siegel, R.L., et al., *Colorectal Cancer in the Young: Epidemiology, Prevention, Management.* American Society of Clinical Oncology Educational Book, 2020(40): p. e75-e88.
20. Araghi, M., et al., *Changes in colorectal cancer incidence in seven high-income countries: a population-based study.* Lancet Gastroenterol Hepatol, 2019. **4**(7): p. 511-518.
21. Siegel, R.L., et al., *Colorectal Cancer Incidence Patterns in the United States, 1974-2013.* J Natl Cancer Inst, 2017. **109**(8).
22. Cao, Y., et al., *Long-term use of antibiotics and risk of colorectal adenoma.* Gut, 2018. **67**(4): p. 672-678.
23. Dik, V.K., et al., *Frequent Use of Antibiotics Is Associated with Colorectal Cancer Risk: Results of a Nested Case-Control Study.* Dig Dis Sci, 2016. **61**(1): p. 255-64.
24. Cho, M., et al., *The interrelationships of the gut microbiome and inflammation in colorectal carcinogenesis.* Clin Lab Med, 2014. **34**(4): p. 699-710.
25. Syngal, S., et al., *ACG Clinical Guideline: Genetic Testing and Management of Hereditary Gastrointestinal Cancer Syndromes.* Official journal of the American College of Gastroenterology | ACG, 2015. **110**(2): p. 223-262.
26. Monahan, K.J., et al., *Guidelines for the management of hereditary colorectal cancer from the British Society of Gastroenterology (BSG)/Association of Coloproctology of Great Britain and Ireland (ACPGBI)/United Kingdom Cancer Genetics Group (UKCGG).* Gut, 2020. **69**(3): p. 411-444.
27. Fijten, G.H., et al., *Predictive value of signs and symptoms for colorectal cancer in patients with rectal bleeding in general practice.* Family Practice, 1995. **12**(3): p. 279-286.
28. Lam, A.K., S.S. Chan, and M. Leung, *Synchronous colorectal cancer: clinical, pathological and molecular implications.* World J Gastroenterol, 2014. **20**(22): p. 6815-20.
29. Beets-Tan, R.G., et al., *Magnetic resonance imaging for clinical management of rectal cancer: updated recommendations from the 2016 European Society of Gastrointestinal and Abdominal Radiology (ESGAR) consensus meeting.* European radiology, 2018. **28**: p. 1465-1475.
30. Nerad, E., et al., *Diagnostic accuracy of CT for local staging of colon cancer: a systematic review and meta-analysis.* American Journal of Roentgenology, 2016. **207**(5): p. 984-995.
31. Benson, A.B., et al., *NCCN guidelines insights: colon cancer, version 2.2018.* Journal of the National Comprehensive Cancer Network, 2018. **16**(4): p. 359-369.
32. Kapiteijn, E., et al., *Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer.* New England Journal of Medicine, 2001. **345**(9): p. 638-646.
33. Engelen, S., et al., *Modern multidisciplinary treatment of rectal cancer based on staging with magnetic resonance imaging leads to excellent local control, but distant control remains a challenge.* European journal of cancer, 2013. **49**(10): p. 2311-2320.
34. Group, M.S., *Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study.* Bmj, 2006. **333**(7572): p. 779.
35. Panzironi, G., et al., *Preoperative locoregional staging of rectal carcinoma: comparison of MR, TRUS and Multislice CT. Personal experience.* La Radiologia Medica, 2004. **107**(4): p. 344-355.
36. van der Paardt, M.P., et al., *Patients who undergo preoperative chemoradiotherapy for locally advanced rectal cancer restaged by using diagnostic MR imaging: a systematic review and meta-analysis.* Radiology, 2013. **269**(1): p. 101-112.
37. Maas, M., et al., *Assessment of clinical complete response after chemoradiation for rectal cancer with digital rectal examination, endoscopy, and MRI: selection for organ-saving treatment.* Annals of surgical oncology, 2015. **22**: p. 3873-3880.
38. Lahaye, M.J., et al., *Locally advanced rectal cancer: MR imaging for restaging after neoadjuvant radiation therapy with concomitant chemotherapy part II. What are the criteria to predict involved lymph nodes?* Radiology, 2009. **252**(1): p. 81-91.
39. Ogura, A., et al., *Neoadjuvant (chemo) radiotherapy with total mesorectal excision only is not sufficient to prevent lateral local recurrence in enlarged nodes: results of the*

multicenter lateral node study of patients with low cT3/4 rectal cancer. Journal of Clinical Oncology, 2019. **37**(1): p. 33.
40. Collaboration, G.B.o.D.C., *The Global Burden of Cancer 2013.* JAMA Oncology, 2015. **1**(4): p. 505-527.
41. Maida, M., et al., *Screening of colorectal cancer: present and future.* Expert Rev Anticancer Ther, 2017. **17**(12): p. 1131-1146.
42. Mandel, J.S., et al., *Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study.* N Engl J Med, 1993. **328**(19): p. 1365-71.
43. Lee, J.K., et al., *Accuracy of fecal immunochemical tests for colorectal cancer: systematic review and meta-analysis.* Ann Intern Med, 2014. **160**(3): p. 171.
44. van Rossum, L.G., et al., *Random comparison of guaiac and immunochemical fecal occult blood tests for colorectal cancer in a screening population.* Gastroenterology, 2008. **135**(1): p. 82-90.
45. Navarro, M., et al., *Colorectal cancer population screening programs worldwide in 2016: An update.* World J Gastroenterol, 2017. **23**(20): p. 3632-3642.
46. Elmunzer, B.J., et al., *Effect of flexible sigmoidoscopy-based screening on incidence and mortality of colorectal cancer: a systematic review and meta-analysis of randomized controlled trials.* PLoS Med, 2012. **9**(12): p. e1001352.
47. Rembacken, B., et al., *Quality in screening colonoscopy: position statement of the European Society of Gastrointestinal Endoscopy (ESGE).* Endoscopy, 2012. **44**(10): p. 957-68.
48. Stoop, E.M., et al., *Participation and yield of colonoscopy versus non-cathartic CT colonography in population-based screening for colorectal cancer: a randomised controlled trial.* Lancet Oncol, 2012. **13**(1): p. 55-64.
49. Bettington, M., et al., *The serrated pathway to colorectal carcinoma: current concepts and challenges.* Histopathology, 2013. **62**(3): p. 367-386.
50. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* Cell, 1990. **61**(5): p. 759-67.
51. Markowitz, S., et al., *Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability.* Science, 1995. **268**(5215): p. 1336-8.
52. Samuels, Y., et al., *High frequency of mutations of the PIK3CA gene in human cancers.* Science, 2004. **304**(5670): p. 554.
53. Baker, S.J., et al., *Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas.* Science, 1989. **244**(4901): p. 217-21.
54. Network, C.G.A., *Comprehensive molecular characterization of human colon and rectal cancer.* Nature, 2012. **487**(7407): p. 330-7.
55. Nguyen, H.T. and H.Q. Duong, *The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy.* Oncol Lett, 2018. **16**(1): p. 9-18.
56. Sinicrope, F.A. and D.J. Sargent, *Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications.* Clin Cancer Res, 2012. **18**(6): p. 1506-12.
57. East, J.E., et al., *British Society of Gastroenterology position statement on serrated polyps in the colon and rectum.* Gut, 2017. **66**(7): p. 1181-1196.
58. Nguyen, H.T. and H.Q. Duong, *The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy (Review).* Oncol Lett, 2018. **16**(1): p. 9-18.
59. Jass, J.R., *Classification of colorectal cancer based on correlation of clinical, morphological and molecular features.* Histopathology, 2007. **50**(1): p. 113-130.
60. Nguyen, L.H., A. Goel, and D.C. Chung, *Pathways of Colorectal Carcinogenesis.* Gastroenterology, 2020. **158**(2): p. 291-302.
61. Guinney, J., et al., *The consensus molecular subtypes of colorectal cancer.* Nature Medicine, 2015. **21**(11): p. 1350-1356.
62. Tamas, K., et al., *Rectal and colon cancer: Not just a different anatomic site.* Cancer Treatment Reviews, 2015. **41**(8): p. 671-679.

63. Hugen, N., et al., *Metastatic pattern in colorectal cancer is strongly influenced by histological subtype.* Annals of Oncology, 2014. **25**(3): p. 651-657.
64. Bertario, L., et al., *Survival of patients with hereditary colorectal cancer: comparison of HNPCC and colorectal cancer in FAP patients with sporadic colorectal cancer.* International journal of cancer, 1999. **80**(2): p. 183-187.
65. Stadler, Z.K., et al., *Characterization of rectal cancer in patients with Lynch syndrome*. 2013, American Society of Clinical Oncology.
66. Fearon, E.R. and B. Vogelstein, *A genetic model for colorectal tumorigenesis.* cell, 1990. **61**(5): p. 759-767.
67. Fodde, R., et al., *Mutations in the APC tumour suppressor gene cause chromosomal instability.* Nature cell biology, 2001. **3**(4): p. 433-438.
68. Van Cutsem, E., et al., *Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer.* New England Journal of Medicine, 2009. **360**(14): p. 1408-1417.
69. Di Nicolantonio, F., et al., *Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer.* Journal of clinical oncology, 2008. **26**(35): p. 5705-5712.
70. Young, J., et al., *Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis.* The American journal of pathology, 2001. **159**(6): p. 2107-2116.
71. Yamauchi, M., et al., *Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum.* Gut, 2012. **61**(6): p. 847-854.
72. Missiaglia, E., et al., *Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features.* Annals of oncology, 2014. **25**(10): p. 1995-2001.
73. Keller, D.S., et al., *The multidisciplinary management of rectal cancer.* Nature Reviews Gastroenterology & Hepatology, 2020. **17**(7): p. 414-429.
74. NCCN. *Clinical Practice Guidelines in Oncology (NCCN Guidelines®). Rectal Cancer, Version 3.2017*. 2017 [cited 2023 01/07/2023]; Available from: https://www.nccn.org/professionals/physician_gls/pdf/rectal.pdf.
75. Backes, Y., et al., *Multicentre prospective evaluation of real-time optical diagnosis of T1 colorectal cancer in large non-pedunculated colorectal polyps using narrow band imaging (the OPTICAL study).* Gut, 2019. **68**(2): p. 271-279.
76. Sano, Y., et al., *Narrow-band imaging (NBI) magnifying endoscopic classification of colorectal tumors proposed by the Japan NBI Expert Team.* Digestive Endoscopy, 2016. **28**(5): p. 526-533.
77. Hayashi, N., et al., *Endoscopic prediction of deep submucosal invasive carcinoma: validation of the narrow-band imaging international colorectal endoscopic (NICE) classification.* Gastrointestinal endoscopy, 2013. **78**(4): p. 625-632.
78. Garcia-Aguilar, J., et al., *Organ preservation for clinical T2N0 distal rectal cancer using neoadjuvant chemoradiotherapy and local excision (ACOSOG Z6041): results of an open-label, single-arm, multi-institutional, phase 2 trial.* The Lancet Oncology, 2015. **16**(15): p. 1537-1546.
79. Rombouts, A.J.M., et al., *Can we Save the rectum by watchful waiting or TransAnal microsurgery following (chemo) Radiotherapy versus Total mesorectal excision for early REctal Cancer (STAR-TREC study)?: protocol for a multicentre, randomised feasibility study.* BMJ Open, 2017. **7**(12): p. e019474.
80. Nahas, S.C., et al., *Diagnostic performance of magnetic resonance to assess treatment response after neoadjuvant therapy in patients with locally advanced rectal cancer.* Abdom Radiol (NY), 2019.
81. Heald, R.J., et al., *Rectal cancer: the Basingstoke experience of total mesorectal excision, 1978-1997.* Archives of surgery, 1998. **133**(8): p. 894-898.

82. Chude, G., et al., *Defunctioning loop ileostomy with low anterior resection for distal rectal cancer: should we make an ileostomy as a routine procedure? A prospective randomized study.* Hepato-gastroenterology, 2008. **55**(86-87): p. 1562-1567.
83. Branda, M.E., et al., *Disease free survival and local recurrence for laparoscopic resection compared to open resection of stage II-III rectal cancer: follow up results of the ACOSOG Z6051 randomized controlled trial.* Annals of surgery, 2019. **269**(4): p. 589.
84. Stevenson, A.R., et al., *Disease-free survival and local recurrence after laparoscopic-assisted resection or open resection for rectal cancer: the Australasian laparoscopic cancer of the rectum randomized clinical trial.* Annals of surgery, 2019. **269**(4): p. 596-602.
85. Jayne, D., et al., *Effect of robotic-assisted vs conventional laparoscopic surgery on risk of conversion to open laparotomy among patients undergoing resection for rectal cancer: the ROLARR randomized clinical trial.* Jama, 2017. **318**(16): p. 1569-1580.
86. McCall, J., M. Cox, and D. Wattchow, *Analysis of local recurrence rates after surgery alone for rectal cancer.* International journal of colorectal disease, 1995. **10**: p. 126-132.
87. Fisher, B., et al., *Postoperative adjuvant chemotherapy or radiation therapy for rectal cancer: results from NSABP protocol R-011.* JNCI: Journal of the National Cancer Institute, 1988. **80**(1): p. 21-29.
88. NICE. *National Institute for Health and Care Excellence: Guidelines Colorectal Cancer*. Colorectal cancer 2021; Available from: https://www.nice.org.uk/guidance/ng151.
89. Bujko, K., et al., *Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer.* Journal of British Surgery, 2006. **93**(10): p. 1215-1223.
90. Probst, C.P., et al., *Extended intervals after neoadjuvant therapy in locally advanced rectal cancer: the key to improved tumor response and potential organ preservation.* Journal of the American College of Surgeons, 2015. **221**(2): p. 430-440.
91. Fokas, E., 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).
92. Smith, J.J., et al., *Assessment of a Watch-and-Wait Strategy for Rectal Cancer in Patients With a Complete Response After Neoadjuvant Therapy.* JAMA Oncology, 2019. **5**(4): p. e185896-e185896.
93. Renehan, A.G., 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.* Lancet Oncol, 2016. **17**(2): p. 174-183.
94. Cunningham, D., et al., *Colorectal cancer.* Lancet, 2010. **375**(9719): p. 1030-47.
95. Feeney, G., et al., *Neoadjuvant radiotherapy for rectal cancer management.* World J Gastroenterol, 2019. **25**(33): p. 4850-4869.
96. Working, M.R.C.R.C., *Randomised trial of surgery alone versus radiotherapy followed by surgery for potentially operable locally advanced rectal cancer.* The Lancet, 1996. **348**(9042): p. 1605-1610.
97. Folkesson, J., et al., *Swedish Rectal Cancer Trial: long lasting benefits from radiotherapy on survival and local recurrence rate.* J Clin Oncol, 2005. **23**(24): p. 5644-5650.
98. Ngan, S.Y., 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.* J Clin Oncol, 2012. **30**(31): p. 3827-33.
99. Sauer, R., et al., *Preoperative versus postoperative chemoradiotherapy for rectal cancer.* N Engl J Med, 2004. **351**(17): p. 1731-40.
100. Sebag-Montefiore, D., et al., *Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial.* The Lancet, 2009. **373**(9666): p. 811-820.
101. O'Connell, M.J., 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): p. 1927.

102. Glynne-Jones, R., et al., *Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Annals of Oncology, 2017. **28**: p. iv22-iv40.
103. You, Y.N., et al., *The American Society of Colon and Rectal Surgeons Clinical Practice Guidelines for the Management of Rectal Cancer.* Dis Colon Rectum, 2020. **63**(9): p. 1191-1222.
104. Benson, A.B., et al., *Rectal Cancer, Version 2.2018, NCCN Clinical Practice Guidelines in Oncology.* J Natl Compr Canc Netw, 2018. **16**(7): p. 874-901.
105. Kasi, A., et al., *Total Neoadjuvant Therapy vs Standard Therapy in Locally Advanced Rectal Cancer: A Systematic Review and Meta-analysis.* JAMA Network Open, 2020. **3**(12): p. e2030097-e2030097.
106. Liu, S., et al., *Total Neoadjuvant Therapy (TNT) versus Standard Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer: A Systematic Review and Meta-Analysis.* Oncologist, 2021. **26**(9): p. e1555-e1566.
107. Fernandez-Martos, C., et al., *Chemoradiation, surgery and adjuvant chemotherapy versus induction chemotherapy followed by chemoradiation and surgery: long-term results of the Spanish GCR-3 phase II randomized trial†.* Ann Oncol, 2015. **26**(8): p. 1722-8.
108. Fokas, E., et al., *Chemoradiotherapy Plus Induction or Consolidation Chemotherapy as Total Neoadjuvant Therapy for Patients With Locally Advanced Rectal Cancer: Long-term Results of the CAO/ARO/AIO-12 Randomized Clinical Trial.* JAMA Oncology, 2022. **8**(1): p. e215445-e215445.
109. Larkin, J., F.S. Hodi, and J.D. Wolchok, *Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma.* N Engl J Med, 2015. **373**(13): p. 1270-1.
110. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* N Engl J Med, 2010. **363**(8): p. 711-23.
111. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation.* J Exp Med, 2000. **192**(7): p. 1027-34.
112. Rizvi, N.A., et al., *Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer.* Science, 2015. **348**(6230): p. 124-8.
113. Masucci, G.V., et al., *Validation of biomarkers to predict response to immunotherapy in cancer: Volume I - pre-analytical and analytical validation.* J Immunother Cancer, 2016. **4**: p. 76.
114. Meng, X., et al., *Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy.* Cancer Treat Rev, 2015. **41**(10): p. 868-76.
115. Llosa, N.J., et al., *The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints.* Cancer Discov, 2015. **5**(1): p. 43-51.
116. Xiao, Y. and G.J. Freeman, *The microsatellite instable subset of colorectal cancer is a particularly good candidate for checkpoint blockade immunotherapy.* Cancer Discov, 2015. **5**(1): p. 16-8.
117. Michael-Robinson, J.M., et al., *Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status.* Gut, 2001. **48**(3): p. 360-6.
118. Taube, J.M., et al., *Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy.* Clin Cancer Res, 2014. **20**(19): p. 5064-74.
119. Le, D.T., et al., *PD-1 Blockade in Tumors with Mismatch-Repair Deficiency.* N Engl J Med, 2015. **372**(26): p. 2509-20.
120. Overman, M.J., et al., *Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study.* Lancet Oncol, 2017. **18**(9): p. 1182-1191.
121. de Rosa, N., et al., *DNA Mismatch Repair Deficiency in Rectal Cancer: Benchmarking Its Impact on Prognosis, Neoadjuvant Response Prediction, and Clinical Cancer Genetics.* J Clin Oncol, 2016. **34**(25): p. 3039-46.

122. Shahabi, V., et al., *Immune-priming of the tumor microenvironment by radiotherapy: rationale for combination with immunotherapy to improve anticancer efficacy.* Am J Clin Oncol, 2015. **38**(1): p. 90-7.
123. Kang, Y.K., et al., *Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial.* Lancet, 2017. **390**(10111): p. 2461-2471.
124. Kato, K., et al., *Nivolumab versus chemotherapy in patients with advanced oesophageal squamous cell carcinoma refractory or intolerant to previous chemotherapy (ATTRACTION-3): a multicentre, randomised, open-label, phase 3 trial.* The Lancet Oncology, 2019. **20**(11): p. 1506-1517.
125. Chalabi, M., et al., *Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers.* Nature Medicine, 2020. **26**(4): p. 566-576.
126. Sharabi, A.B., et al., *Stereotactic Radiation Therapy Augments Antigen-Specific PD-1-Mediated Antitumor Immune Responses via Cross-Presentation of Tumor Antigen.* Cancer Immunol Res, 2015. **3**(4): p. 345-55.
127. Bando, H., et al., *Novel Immunological Approaches in the Treatment of Locally Advanced Rectal Cancer.* Clinical Colorectal Cancer, 2022. **21**(1): p. 3-9.
128. Habr-Gama, A., et al., *Complete clinical response after neoadjuvant chemoradiation therapy for distal rectal cancer: characterization of clinical and endoscopic findings for standardization.* Diseases of the colon & rectum, 2010. **53**(12): p. 1692-1698.
129. Habr-Gama, A., et al., *Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results.* Annals of surgery, 2004. **240**(4): p. 711.
130. Martens, M.H., et al., *Long-term outcome of an organ preservation program after neoadjuvant treatment for rectal cancer.* JNCI: Journal of the National Cancer Institute, 2016. **108**(12): p. djw171.
131. Chadi, S.A., et al., *Factors affecting local regrowth after watch and wait for patients with a clinical complete response following chemoradiotherapy in rectal cancer (InterCoRe consortium): an individual participant data meta-analysis.* The lancet Gastroenterology & hepatology, 2018. **3**(12): p. 825-836.
132. van der Valk, M.J., 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): p. 2537-2545.
133. Gao, Y., et al., *Oxaliplatin and capecitabine concomitant with neoadjuvant radiotherapy and extended to the resting period in high risk locally advanced rectal cancer.* Strahlentherapie und Onkologie, 2014. **190**(2): p. 158.
134. São Julião, G.P., et al., *Is neoadjuvant chemoradiation with dose-escalation and consolidation chemotherapy sufficient to increase surgery-free and distant metastases-free survival in baseline cT3 rectal cancer?* European Journal of Surgical Oncology, 2018. **44**(1): p. 93-99.
135. Asoglu, O., et al., *The impact of total neo-adjuvant treatment on nonoperative management in patients with locally advanced rectal cancer: The evaluation of 66 cases.* European Journal of Surgical Oncology, 2020. **46**(3): p. 402-409.
136. Lambregts, D.M., et al., *Diffusion-weighted MRI to assess response to chemoradiotherapy in rectal cancer: main interpretation pitfalls and their use for teaching.* European Radiology, 2017. **27**: p. 4445-4454.
137. Perez, R.O., et al., *Optimal timing for assessment of tumor response to neoadjuvant chemoradiation in patients with rectal cancer: do all patients benefit from waiting longer than 6 weeks?* International Journal of Radiation Oncology* Biology* Physics, 2012. **84**(5): p. 1159-1165.

138. Cerdán-Santacruz, C., et al., *Watch and wait: Why, to whom and how.* Surgical Oncology, 2022. **43**: p. 101774.
139. Habr-Gama, A., et al., *Achieving a Complete Clinical Response After Neoadjuvant Chemoradiation That Does Not Require Surgical Resection: It May Take Longer Than You Think!* Diseases of the Colon & Rectum, 2019. **62**(7): p. 802-808.
140. Deidda, S., et al., *Association of Delayed Surgery With Oncologic Long-term Outcomes in Patients With Locally Advanced Rectal Cancer Not Responding to Preoperative Chemoradiation.* JAMA Surgery, 2021. **156**(12): p. 1141-1149.
141. Dattani, M., et al., *Oncological and Survival Outcomes in Watch and Wait Patients With a Clinical Complete Response After Neoadjuvant Chemoradiotherapy for Rectal Cancer: A Systematic Review and Pooled Analysis.* Annals of Surgery, 2018. **268**(6): p. 955-967.
142. Garcia-Aguilar, J., S. Patil, and M. Gollub, *Organ preservation in patients with rectal adenocarcinoma treated with total neoadjuvant therapy.* J Clin Oncol, 2022. **40**: p. 2546-2556.
143. Benson, A.B., et al., *Rectal Cancer, Version 2.2022, NCCN Clinical Practice Guidelines in Oncology.* J Natl Compr Canc Netw, 2022. **20**(10): p. 1139-1167.
144. Rojo, A., et al., *Update on the surgical pathology standards on rectal cancer diagnosis, staging and quality assessment of surgery.* Clinical and Translational Oncology, 2010. **12**(6): p. 431-436.
145. Hermanek, P., S. Merkel, and W. Hohenberger, *Prognosis of rectal carcinoma after multimodal treatment: ypTNM classification and tumor regression grading are essential.* Anticancer Res, 2013. **33**(2): p. 559-66.
146. Rödel, C., et al., *Prognostic Significance of Tumor Regression After Preoperative Chemoradiotherapy for Rectal Cancer.* Journal of Clinical Oncology, 2005. **23**(34): p. 8688-8696.
147. Dworak, O., L. Keilholz, and A. Hoffmann, *Pathological features of rectal cancer after preoperative radiochemotherapy.* International Journal of Colorectal Disease, 1997. **12**(1): p. 19-23.
148. Wheeler, J.M.D., et al., *Quantification of Histologic Regression of Rectal Cancer After Irradiation: A Proposal for a Modified Staging System.* Diseases of the Colon & Rectum, 2002. **45**(8): p. 1051-1056.
149. Mandard, A.-M., et al., *Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations.* Cancer, 1994. **73**(11): p. 2680-2686.
150. Ryan, R., et al., *Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer.* Histopathology, 2005. **47**(2): p. 141-146.
151. Ryan, J.E., et al., *Assessing pathological complete response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer: a systematic review.* Colorectal Disease, 2015. **17**(10): p. 849-861.
152. Patel, U.B., et al., *MRI After Treatment of Locally Advanced Rectal Cancer: How to Report Tumor Response—The MERCURY Experience.* American Journal of Roentgenology, 2012. **199**(4): p. W486-W495.
153. Patel, U.B., et al., *Magnetic Resonance Imaging–Detected Tumor Response for Locally Advanced Rectal Cancer Predicts Survival Outcomes: MERCURY Experience.* Journal of Clinical Oncology, 2011. **29**(28): p. 3753-3760.
154. Konishi, T., et al., *Association of Preoperative and Postoperative Serum Carcinoembryonic Antigen and Colon Cancer Outcome.* JAMA Oncol, 2018. **4**(3): p. 309-315.
155. Wallin, U., et al., *CEA - a predictor for pathologic complete response after neoadjuvant therapy for rectal cancer.* Dis Colon Rectum, 2013. **56**(7): p. 859-68.
156. Park, Y.-A., et al., *Serum CEA as a predictor for the response to preoperative chemoradiation in rectal cancer.* Journal of Surgical Oncology, 2006. **93**(2): p. 145-150.

157. George, T.J., Jr., C.J. Allegra, and G. Yothers, *Neoadjuvant Rectal (NAR) Score: a New Surrogate Endpoint in Rectal Cancer Clinical Trials.* Curr Colorectal Cancer Rep, 2015. **11**(5): p. 275-280.
158. André, T., et al., *Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial.* J clin oncol, 2009. **27**(19): p. 3109-3116.
159. Haller, D.G., et al., *Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer.* J Clin Oncol, 2011. **29**(11): p. 1465-1471.
160. Kuebler, J.P., et al., *Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07.* Journal of clinical oncology, 2007. **25**(16): p. 2198-2204.
161. Yothers, G., et al., *Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses.* Journal of clinical oncology, 2011. **29**(28): p. 3768.
162. Braendengen, M., et al., *Randomized phase III study comparing preoperative radiotherapy with chemoradiotherapy in nonresectable rectal cancer.* J Clin Oncol, 2008. **26**(22): p. 3687-94.
163. Iveson, T.J., et al., *3 versus 6 months of adjuvant oxaliplatin-fluoropyrimidine combination therapy for colorectal cancer (SCOT): an international, randomised, phase 3, non-inferiority trial.* The Lancet Oncology, 2018. **19**(4): p. 562-578.
164. André, T., et al., *Effect of duration of adjuvant chemotherapy for patients with stage III colon cancer (IDEA collaboration): final results from a prospective, pooled analysis of six randomised, phase 3 trials.* The Lancet Oncology, 2020. **21**(12): p. 1620-1629.
165. Breugom, A.J., et al., *Adjuvant chemotherapy after preoperative (chemo)radiotherapy and surgery for patients with rectal cancer: a systematic review and meta-analysis of individual patient data.* The Lancet Oncology, 2015. **16**(2): p. 200-207.
166. Gustavsson, B., et al., *A Review of the Evolution of Systemic Chemotherapy in the Management of Colorectal Cancer.* Clinical Colorectal Cancer, 2015. **14**(1): p. 1-10.
167. Tuomisto, A.E., M.J. Mäkinen, and J.P. Väyrynen, *Systemic inflammation in colorectal cancer: Underlying factors, effects, and prognostic significance.* World J Gastroenterol, 2019. **25**(31): p. 4383-4404.
168. McAllister, S.S. and R.A. Weinberg, *The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis.* Nat Cell Biol, 2014. **16**(8): p. 717-27.
169. Roxburgh, C.S. and D.C. McMillan, *Role of systemic inflammatory response in predicting survival in patients with primary operable cancer.* Future Oncol, 2010. **6**(1): p. 149-63.
170. Guthrie, G.J.K., et al., *The systemic inflammation-based neutrophil–lymphocyte ratio: Experience in patients with cancer.* Critical Reviews in Oncology/Hematology, 2013. **88**(1): p. 218-230.
171. Roxburgh, C.S.D., et al., *Comparison of the Prognostic Value of Inflammation-Based Pathologic and Biochemical Criteria in Patients Undergoing Potentially Curative Resection for Colorectal Cancer.* Annals of Surgery, 2009. **249**(5).
172. Dolan, R.D., et al., *The role of the systemic inflammatory response in predicting outcomes in patients with advanced inoperable cancer: Systematic review and meta-analysis.* Crit Rev Oncol Hematol, 2017. **116**: p. 134-146.
173. Watt, D.G., et al., *Neutrophil count is the most important prognostic component of the differential white cell count in patients undergoing elective surgery for colorectal cancer.* The American Journal of Surgery, 2015. **210**(1): p. 24-30.
174. Di Carlo, E., G. Forni, and P. Musiani, *Neutrophils in the antitumoral immune response.* Chem Immunol Allergy, 2003. **83**: p. 182-203.
175. Fridlender, Z.G. and S.M. Albelda, *Tumor-associated neutrophils: friend or foe?* Carcinogenesis, 2012. **33**(5): p. 949-55.

176. Kusumanto, Y.H., et al., *Platelets and granulocytes, in particular the neutrophils, form important compartments for circulating vascular endothelial growth factor.* Angiogenesis, 2003. **6**(4): p. 283-7.
177. Mabuchi, S., et al., *Uterine cervical cancer displaying tumor-related leukocytosis: a distinct clinical entity with radioresistant feature.* J Natl Cancer Inst, 2014. **106**(7).
178. Shamamian, P., et al., *Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis.* J Cell Physiol, 2001. **189**(2): p. 197-206.
179. Tsai, P.-L., et al., *Neutrophil–lymphocyte ratio and CEA level as prognostic and predictive factors in colorectal cancer: A systematic review and meta-analysis.* Journal of Cancer Research and Therapeutics, 2016. **12**(2).
180. Kim, T.G., et al., *Effect of leukocyte alteration on treatment outcomes following preoperative chemoradiotherapy in patients with rectal cancer.* Radiat Oncol J, 2017. **35**(3): p. 217-226.
181. Ishikawa, D., et al., *The Role of Neutrophil-to-lymphocyte Ratio on the Effect of CRT for Patients With Rectal Cancer.* In Vivo, 2020. **34**(2): p. 863-868.
182. Braun, L.H., et al., *Neutrophil-to-Lymphocyte Ratio in Rectal Cancer-Novel Biomarker of Tumor Immunogenicity During Radiotherapy or Confounding Variable?* Int J Mol Sci, 2019. **20**(10).
183. Zhang, X., et al., *Association of markers of systemic and local inflammation with prognosis of patients with rectal cancer who received neoadjuvant radiotherapy.* Cancer Manag Res, 2019. **11**: p. 191-199.
184. Shen, L., et al., *Baseline neutrophil-lymphocyte ratio (⩾2.8) as a prognostic factor for patients with locally advanced rectal cancer undergoing neoadjuvant chemoradiation.* Radiation Oncology, 2014. **9**(1): p. 295.
185. Venkatesulu, B.P., et al., *A systematic review of the influence of radiation-induced lymphopenia on survival outcomes in solid tumors.* Critical Reviews in Oncology/Hematology, 2018. **123**: p. 42-51.
186. Campian, J.L., et al., *Severe Treatment-Related Lymphopenia in Patients with Newly Diagnosed Rectal Cancer.* Cancer Investigation, 2018. **36**(6): p. 356-361.
187. Heo, J., et al., *Sustaining Blood Lymphocyte Count during Preoperative Chemoradiotherapy as a Predictive Marker for Pathologic Complete Response in Locally Advanced Rectal Cancer.* Cancer Res Treat, 2016. **48**(1): p. 232-239.
188. Beard, J.L., *Iron biology in immune function, muscle metabolism and neuronal functioning.* The Journal of nutrition, 2001. **131**(2): p. 568S-580S.
189. Rieger, M.A. and T. Schroeder, *Hematopoiesis.* Cold Spring Harbor perspectives in biology, 2012. **4**(12): p. a008250.
190. Shvartsman, M., S. Bilican, and C. Lancrin, *Iron deficiency disrupts embryonic haematopoiesis but not the endothelial to haematopoietic transition.* Scientific Reports, 2019. **9**(1): p. 6414.
191. Phipps, O., M.J. Brookes, and H.O. Al-Hassi, *Iron deficiency, immunology, and colorectal cancer.* Nutrition Reviews, 2020. **79**(1): p. 88-97.
192. Kramer, J.L., et al., *Differentiation of functional dendritic cells and macrophages from human peripheral blood monocyte precursors is dependent on expression of p21 (WAF1/CIP1) and requires iron.* British journal of haematology, 2002. **117**(3): p. 727-734.
193. Gafter-Gvili, A., et al., *Oxidative stress-induced DNA damage and repair in human peripheral blood mononuclear cells: protective role of hemoglobin.* PLoS One, 2013. **8**(7): p. e68341.
194. Aslan, M., et al., *Lymphocyte DNA damage and oxidative stress in patients with iron deficiency anemia.* Mutation research/fundamental and molecular mechanisms of mutagenesis, 2006. **601**(1-2): p. 144-149.
195. Baginska, J., et al., *Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia.* Proceedings of the National Academy of Sciences, 2013. **110**(43): p. 17450-17455.

196. Bergman, M., et al., *In vitro cytokine production in patients with iron deficiency anemia.* Clinical Immunology, 2004. **113**(3): p. 340-344.
197. Evans, C., et al., *The correlation between colorectal cancer rates of proliferation and apoptosis and systemic cytokine levels; plus their influence upon survival.* British journal of cancer, 2006. **94**(10): p. 1412-1419.
198. Siveen, K. and G. Kuttan, *Role of macrophages in tumour progression.* Immunology letters, 2009. **123**(2): p. 97-102.
199. Nitti, M., et al., *HO-1 induction in cancer progression: a matter of cell adaptation.* Antioxidants, 2017. **6**(2): p. 29.
200. Costa da Silva, M., et al., *Iron induces anti-tumor activity in tumor-associated macrophages.* Frontiers in immunology, 2017. **8**: p. 1479.
201. Handa, P., et al., *Iron alters macrophage polarization status and leads to steatohepatitis and fibrogenesis.* Journal of Leukocyte Biology, 2019. **105**(5): p. 1015-1026.
202. Bonaccorsi-Riani, E., et al., *Iron deficiency impairs intra-hepatic lymphocyte mediated immune response.* PLoS One, 2015. **10**(8): p. e0136106.
203. Quirke, P., *Training and quality assurance for rectal cancer: 20 years of data is enough.* The Lancet Oncology, 2003. **4**(11): p. 695-702.
204. Roh, M.S., et al., *Preoperative multimodality therapy improves disease-free survival in patients with carcinoma of the rectum: NSABP R-03.* J Clin Oncol, 2009. **27**(31): p. 5124-30.
205. Sauer, R., et al., *Preoperative versus Postoperative Chemoradiotherapy for Rectal Cancer.* New England Journal of Medicine, 2004. **351**(17): p. 1731-1740.
206. Sebag-Montefiore, D., et al., *Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial.* Lancet, 2009. **373**(9666): p. 811-820.
207. Jamieson, N.B., et al., *The Relationship Between Tumor Inflammatory Cell Infiltrate and Outcome in Patients with Pancreatic Ductal Adenocarcinoma.* Annals of Surgical Oncology, 2012. **19**(11): p. 3581-3590.
208. Proctor, M.J., et al., *A derived neutrophil to lymphocyte ratio predicts survival in patients with cancer.* British Journal of Cancer, 2012. **107**(4): p. 695-699.
209. Toiyama, Y., et al., *C-reactive protein as predictor of recurrence in patients with rectal cancer undergoing chemoradiotherapy followed by surgery.* Anticancer Res, 2013. **33**(11): p. 5065-74.
210. Carruthers, R., et al., *Systemic inflammatory response is a predictor of outcome in patients undergoing preoperative chemoradiation for locally advanced rectal cancer.* Colorectal Disease, 2012. **14**(10): p. e701-e707.
211. Dreyer, S.B., et al., *The Pretreatment Systemic Inflammatory Response is an Important Determinant of Poor Pathologic Response for Patients Undergoing Neoadjuvant Therapy for Rectal Cancer.* Ann Surg Oncol, 2017. **24**(5): p. 1295-1303.
212. *Systemic Immune-inflammation Index (SII) Predicts Pathological Complete Response to Neoadjuvant Chemoradiotherapy in Locally Advanced Rectal Cancer.* Journal of the College of Physicians and Surgeons Pakistan, 2021. **31**(4): p. 399-404.
213. Kim, T.G., et al., *Baseline neutrophil–lymphocyte ratio and platelet–lymphocyte ratio in rectal cancer patients following neoadjuvant chemoradiotherapy.* Tumori Journal, 2019. **105**(5): p. 434-440.
214. Sawada, R., et al., *Systemic Inflammatory Markers Combined with Tumor-Infiltrating Lymphocyte Density for the Improved Prediction of Response to Neoadjuvant Chemoradiotherapy in Rectal Cancer.* Annals of Surgical Oncology, 2021. **28**(11): p. 6189-6198.
215. Krauthamer, M., et al., *A Study of Inflammation-Based Predictors of Tumor Response to Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer.* Oncology, 2013. **85**(1): p. 27-32.

216. Ryan, R., et al., *Pathological response following long-course neoadjuvant chemoradiotherapy for locally advanced rectal cancer.* Histopathology, 2005. **47**(2): p. 141-6.
217. Royal College of Pathologists, U., *Dataset for histopathological reporting of colorectal cancer* 2018.
218. Proctor, M.J., et al., *An inflammation-based prognostic score (mGPS) predicts cancer survival independent of tumour site: a Glasgow Inflammation Outcome Study.* Br J Cancer, 2011. **104**(4): p. 726-34.
219. Costa-Silva, L. and G. Brown, *Magnetic resonance imaging of rectal cancer.* Magnetic resonance imaging clinics of North America, 2013. **21 2**: p. 385-408.
220. Maas, M., et al., *Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: a pooled analysis of individual patient data.* Lancet Oncol, 2010. **11**(9): p. 835-44.
221. Royal College of Pathologists, U., *Dataset for histopathological reporting of colorectal cancer.* 2018.
222. Perez, R.O., et al., *The Role of Carcinoembriogenic Antigen in Predicting Response and Survival to Neoadjuvant Chemoradiotherapy for Distal Rectal Cancer.* Diseases of the Colon & Rectum, 2009. **52**(6): p. 1137-1143.
223. Li, A., et al., *Pretreatment blood biomarkers predict pathologic responses to neo-CRT in patients with locally advanced rectal cancer.* Future Oncol, 2019. **15**(28): p. 3233-3242.
224. Sawada, R., et al., *Systemic Inflammatory Markers Combined with Tumor-Infiltrating Lymphocyte Density for the Improved Prediction of Response to Neoadjuvant Chemoradiotherapy in Rectal Cancer.* Ann Surg Oncol, 2021. **28**(11): p. 6189-6198.
225. Hamid, H.K.S., et al., *Prognostic and predictive value of neutrophil-to-lymphocyte ratio after curative rectal cancer resection: A systematic review and meta-analysis.* Surgical Oncology, 2021. **37**: p. 101556.
226. Jeon, B.H., et al., *Neutrophil to Lymphocyte Ratio: A Predictive Marker for Treatment Outcomes in Patients With Rectal Cancer Who Underwent Neoadjuvant Chemoradiation Followed by Surgery.* Ann Coloproctol, 2019. **35**(2): p. 100-106.
227. Zhang, Y., et al., *Prognostic value of pretreatment systemic inflammatory markers in patients with locally advanced rectal cancer following neoadjuvant chemoradiotherapy.* Sci Rep, 2020. **10**(1): p. 8017.
228. Motomura, T., et al., *Neutrophil–lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment.* Journal of hepatology, 2013. **58**(1): p. 58-64.
229. Kantola, T., et al., *Stage-dependent alterations of the serum cytokine pattern in colorectal carcinoma.* Br. J. Cancer, 2012. **107**(10): p. 1729-1736.
230. Heys, S.D., et al., *Serum albumin: a prognostic indicator in patients with colorectal cancer.* Journal of the Royal College of Surgeons of Edinburgh, 1998. **43**(3): p. 163-168.
231. Proctor, M.J., et al., *Optimization of the systemic inflammation-based Glasgow Prognostic Score.* Cancer, 2013. **119**(12): p. 2325-2332.
232. McMillan, D.C., et al., *Albumin Concentrations Are Primarily Determined by the Body Cell Mass and the Systemic Inflammatory Response in Cancer Patients With Weight Loss.* Nutrition and Cancer, 2001. **39**(2): p. 210-213.
233. Lai, S., et al., *Systemic inflammatory indices predict tumor response to neoadjuvant chemoradiotherapy for locally advanced rectal cancer.* Oncol Lett, 2020. **20**(3): p. 2763-2770.
234. Moureau-Zabotto, L., et al., *Predictive factors of tumor response after neoadjuvant chemoradiation for locally advanced rectal cancer.* Int J Radiat Oncol Biol Phys, 2011. **80**(2): p. 483-91.
235. Restivo, A., 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): p. 864-871.

236. Probst, C.P., et al., *Watch and Wait?—Elevated Pretreatment CEA Is Associated with Decreased Pathological Complete Response in Rectal Cancer.* Journal of Gastrointestinal Surgery, 2016. **20**(1): p. 43-52.
237. Horvat, N., et al., *MRI of Rectal Cancer: Tumor Staging, Imaging Techniques, and Management.* Radiographics, 2019. **39**(2): p. 367-387.
238. Ménétrier-Caux, C., et al., *Lymphopenia in Cancer Patients and its Effects on Response to Immunotherapy: an opportunity for combination with Cytokines?* Journal for ImmunoTherapy of Cancer, 2019. **7**(1): p. 85.
239. Kitayama, J., et al., *Circulating lymphocyte is an important determinant of the effectiveness of preoperative radiotherapy in advanced rectal cancer.* BMC Cancer, 2011. **11**(1): p. 64.
240. Howell, J.N., et al., *Relationship of lymphopenia to overall survival and progression-free survival in rectal cancer patients receiving neoadjuvant chemoradiation.* Journal of Clinical Oncology, 2020. **38**(4_suppl): p. 39-39.
241. Health, U.D.o. and H. Services, *Common terminology criteria for adverse events. Version 5.0. Published November 27, 2017*. 2020.
242. Heo, J., et al., *Sustaining Blood Lymphocyte Count during Preoperative Chemoradiotherapy as a Predictive Marker for Pathologic Complete Response in Locally Advanced Rectal Cancer.* crt, 2015. **48**(1): p. 232-239.
243. Wei, S.C., C.R. Duffy, and J.P. Allison, *Fundamental Mechanisms of Immune Checkpoint Blockade Therapy.* Cancer Discovery, 2018. **8**(9): p. 1069-1086.
244. Shasha, D., *The negative impact of anemia on radiotherapy and chemoradiation outcomes.* Seminars in Hematology, 2001. **38**: p. 8-15.
245. Hoff, C.M., *Importance of hemoglobin concentration and its modification for the outcome of head and neck cancer patients treated with radiotherapy.* Acta Oncologica, 2012. **51**(4): p. 419-432.
246. Alarcón, R., et al., *Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation.* Cancer Res, 1999. **59**(24): p. 6046-51.
247. Harrison, L.B., et al., *Impact of Tumor Hypoxia and Anemia on Radiation Therapy Outcomes.* The Oncologist, 2002. **7**(6): p. 492-508.
248. Walter, C.J., et al., *Prevalence and significance of anaemia in patients receiving long-course neoadjuvant chemoradiotherapy for rectal carcinoma.* Colorectal Disease, 2013. **15**(1): p. 52-56.
249. McGrane, J.M., et al., *Significance of Anemia in Outcomes After Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer.* Clinical Colorectal Cancer, 2017. **16**(4): p. 381-385.
250. Lee, H., et al., *Negative impact of pretreatment anemia on local control after neoadjuvant chemoradiotherapy and surgery for rectal cancer.* Radiat Oncol J, 2012. **30**(3): p. 117-123.
251. Khan, A.A., et al., *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): p. 1232-7.
252. Lee, S.D., et al., *Influence of anemia on tumor response to preoperative chemoradiotherapy for locally advanced rectal cancer.* International Journal of Colorectal Disease, 2009. **24**(12): p. 1451-1458.
253. Box, B., et al., *Neoadjuvant Therapy for Rectal Cancer: Improved Tumor Response, Local Recurrence, and Overall Survival in Nonanemic Patients.* Diseases of the Colon & Rectum, 2005. **48**(6): p. 1153-1160.
254. Bong, J.W., et al., *Effect of anaemia on the response to preoperative chemoradiotherapy for rectal cancer.* ANZ Journal of Surgery, 2021. **91**(5): p. E286-E291.
255. Rodrigues, D., et al., *Baseline anaemia increases locally advanced rectal cancer mortality in older patients undergoing preoperative chemoradiation.* Supportive Care in Cancer, 2021. **29**(3): p. 1403-1411.

256. Berardi, R., et al., *Anemia may influence the outcome of patients undergoing neo-adjuvant treatment of rectal cancer.* Ann Oncol, 2006. **17**(11): p. 1661-4.
257. Richards, C.H., et al., *Prognostic value of tumour necrosis and host inflammatory responses in colorectal cancer.* British Journal of Surgery, 2011. **99**(2): p. 287-294.
258. McSorley, S.T., et al., *Normocytic anaemia is associated with systemic inflammation and poorer survival in patients with colorectal cancer treated with curative intent.* International Journal of Colorectal Disease, 2019. **34**(3): p. 401-408.
259. Institute, N.C. *National Cancer Institute Common Terminology Criteria for Adverse Event (CTCAE) version 5*. 2017; Available from: https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_5x7.pdf.
260. Nemeth, E. and T. Ganz, *Anemia of inflammation.* Hematol Oncol Clin North Am, 2014. **28**(4): p. 671-81, vi.
261. Devon, K.M. and R.S. McLeod, *Pre and peri-operative erythropoeitin for reducing allogeneic blood transfusions in colorectal cancer surgery.* Cochrane Database of Systematic Reviews, 2009(1).
262. Vaupel, P., *The Role of Hypoxia-Induced Factors in Tumor Progression.* The Oncologist, 2004. **9**(S5): p. 10-17.
263. Harris, A.L., *Hypoxia — a key regulatory factor in tumour growth.* Nature Reviews Cancer, 2002. **2**(1): p. 38-47.
264. Janssen, H.L., et al., *Hypoxia in head and neck cancer: How much, how important?* Head & Neck, 2005. **27**(7): p. 622-638.
265. De Ridder, M., et al., *Macrophages enhance the radiosensitizing activity of lipid A: a novel role for immune cells in tumor cell radioresponse.* International Journal of Radiation Oncology* Biology* Physics, 2004. **60**(2): p. 598-606.
266. De Ridder, M., et al., *Hypoxic tumor cell radiosensitization through nitric oxide.* Nitric Oxide, 2008. **19**(2): p. 164-9.
267. Sermeus, A., et al., *Advances in radiotherapy delivery for rectal cancer: a European perspective.* Expert Review of Gastroenterology & Hepatology, 2015. **9**(4): p. 393-397.
268. Park, J.H., et al., *Preoperative, biopsy-based assessment of the tumour microenvironment in patients with primary operable colorectal cancer.* J Pathol Clin Res, 2020. **6**(1): p. 30-39.
269. Hanna, C.R., et al., *Durvalumab (MEDI 4736) in combination with extended neoadjuvant regimens in rectal cancer: a study protocol of a randomised phase II trial (PRIME-RT).* Radiat Oncol, 2021. **16**(1): p. 163.
270. Verheij, F.S., et al., *Long-Term Results of Organ Preservation in Patients With Rectal Adenocarcinoma Treated With Total Neoadjuvant Therapy: The Randomized Phase II OPRA Trial.* J Clin Oncol, 2024. **42**(5): p. 500-506.
271. Dijkstra, E.A., et al., *Locoregional Failure During and After Short-course Radiotherapy Followed by Chemotherapy and Surgery Compared With Long-course Chemoradiotherapy and Surgery: A 5-Year Follow-up of the RAPIDO Trial.* Ann Surg, 2023. **278**(4): p. e766-e772.
272. Cercek, A., et al., *PD-1 Blockade in Mismatch Repair–Deficient, Locally Advanced Rectal Cancer.* New England Journal of Medicine, 2022. **386**(25): p. 2363-2376.

Appendix I

Table 2.1s. Baseline clinical and pathological of both cohorts used to construct the study dataset. Cohort 1 consists of patients from Glasgow Royal Infirmary dataset. Cohort 2 consists of patients from West of Scotland dataset which also included patients from Glasgow Royal Infirmary. Numbers in parentheses indicate percentages. P values for Chi Square testing.

		Cohort 1	Cohort 2	P Value
Age (years)	<55	11 (19)	33 (19)	0.085
	55 – 74	43 (73)	101 (60)	
	≥75	5 (8)	35 (21)	
Sex	Female	23 (39)	65 (38)	0.944
	Male	36 (61)	104 (62)	
BMI	<25	22 (46)	48 (37)	0.050
	25-30	20 (42)	43 (33)	
	>30	6 (12)	40 (30)	
Tumour Height from anal verge (cm)	<5 (low)	28 (50)	65 (40)	0.435
	5 – 10 (mid)	19 (34)	62 (39)	
	>10 (upper)	9 (16)	34 (21)	
Tumour Size (cm)	<4	3 (6)	32 (19)	0.066
	4-7	44 (83)	119 (71)	
	≥8	6 (11)	17 (10)	
Clinical T Stage	T2	4 (7)	11 (7)	0.962
	T3	44 (75)	129 (76)	
	T4	11 (19)	29 (17)	
Clinical N Stage	N0	23 (39)	41 (24)	0.030
	N1/2	36 (61)	128 (76)	
Clinical TNM stage	I	3 (5)	2 (1)	0.042
	II	20 (34)	39 (23)	

	III	36 (61)	128 (76)	
RT Dose (Gy)	<45	1 (3)	7 (4)	0.716
	45	31 (97)	158 (94)	
	>45 (boost)	0	3 (2)	
Concomitant Chemotherapy	Capecitabine	43 (84)	107 (88)	0.253
	5-FU	8 (16)	11 (9)	
	Other *	0	3 3 (3)	
Interval: CRT finish to Surgery (weeks)	≤8	16 (27)	9 (5)	<0.001
	8-12	32 (54)	96 (67)	
	>12	11 (19)	64 (38)	
Operation	TME with primary anastomosis	26 (44)	60 (36)	0.306
	TME with end colostomy	3 (5)	16 (9)	
	Abdominoperineal resection (APR)	30 (51)	88 (52)	
	Pelvic Exenteration **	0	5 (3)	
Pathological T staging	ypT0	10 (17)	35 (21)	0.685
	ypT1	3 (5)	9 (5)	
	ypT2	10 (17)	30 (18)	
	ypT3	34 (58)	82 (49)	
	ypT4	2 (3)	13 (8)	
Pathological N staging	ypN0	41 (69)	122 (72)	0.059
	ypN1	10 (17)	39 (23)	
	ypN2	8 (14)	9 (5)	
Pathological TNM stage	0	10 (17)	35 (21)	0.823
	I	3 (5)	6 (4)	
	II	27 (46)	81 (48)	

	III	19 (32)	47 (28)	
pCR	No	49 (83)	134 (79)	0.532
	Yes	10 (17)	35 (21)	
NAR score	<8 (low risk)	12 (20)	40 (24)	0.870
	8-16 (intermediate risk)	29 (49)	79 (47)	
	>16 (high risk)	18 (31)	50 (3)	
Tumour Regression Grade	TRG 0 (pCR)	10 (17)	35 (22)	0.138
	TRG 1	11 (19)	24 (15)	
	TRG 2	29 (49)	56 (35)	
	TRG 3	9 (15)	43 (27)	
Resection Margin	R0	51 (86)	148 (88)	0.822
	R1 (≤1mm to CRM)	8 (14)	21 (12)	

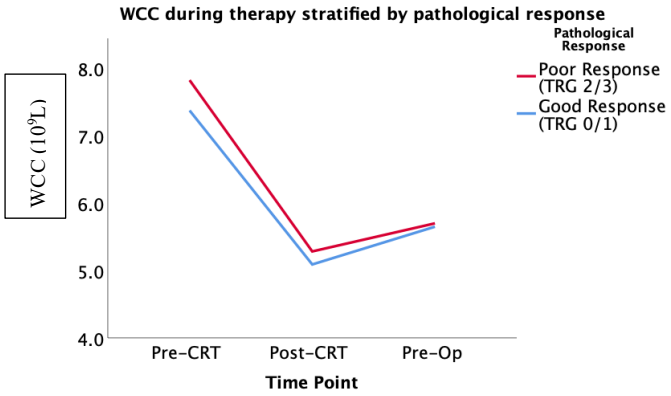
Table 2.8s. Median values of white cell count, its constituents, NLR, C-Reactive protein and albumin over time P values Mann-Whitney U test comparing good and poor pathological response at each time point.

	Timepoint	**Tumour regression grade**		**p value**
		Good (TRG 0/1)	**Poor (TRG 2/3)**	
WCC (10^9/L)	Pre-CRT	7.23	7.91	0.272
	Post-CRT	4.37	5.30	0.580
	Pre-Op	5.60	5.50	0.759
Neutrophils (10^9/L)	Pre-CRT	4.46	5.10	0.088
	Post-CRT	3.14	3.50	0.560
	Pre-Op	3.14	3.50	0.784
Lymphocytes (10^9/L)	Pre-CRT	1.83	1.66	0.125
	Post-CRT	0.52	0.69	0.452
	Pre-Op	0.80	0.90	0.316
NLR	Pre-CRT	2.82	2.86	0.087
	Post-CRT	5.89	4.79	0.394
	Pre-Op	4.30	4.08	0.655
CRP (mg/L)	Pre-CRT	3.00	3.75	0.284
	Post-CRT	2.20	3.00	0.071
	Pre-Op	2.40	3.05	0.366
Albumin	Pre-CRT	37	38	0.199
	Post-CRT	36	36	0.022
	Pre-Op	37	37	0.015

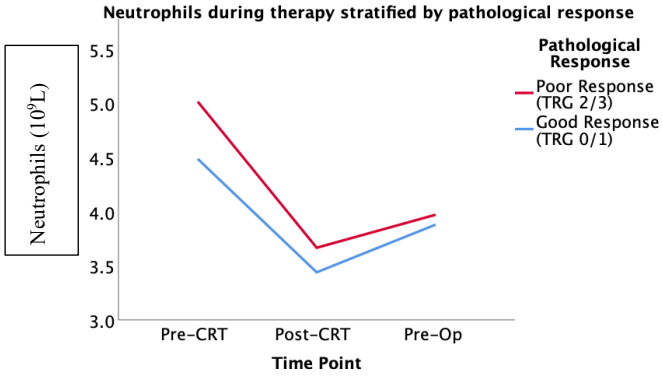
CRT chemoradiotherapy; WBC total white cell count; NLR neutrophil to lymphocyte ratio; CRP C-reactive protein.

Figures 2.5s Median values of serum markers prior to and following neoadjuvant therapy stratified by pathological tumour regression grade (TRG). TRG 0/1 indicates good response and TRG 2

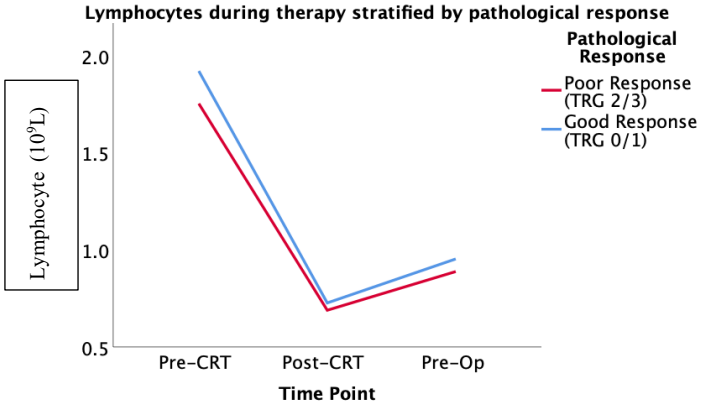
2.5.1s White cell count (WCC)



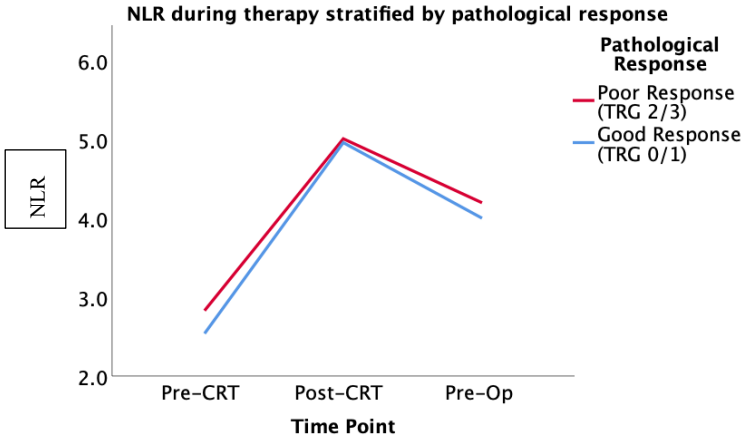
2.5.2s Neutrophil count



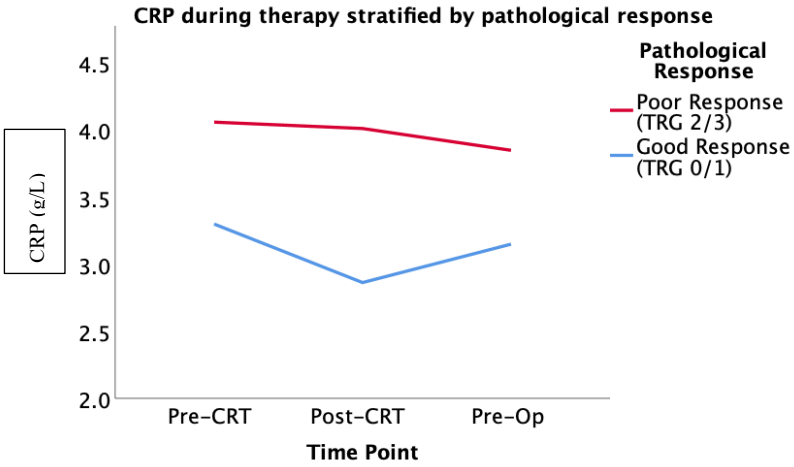
2.5.3s Lymphocyte count



2.5.4s Neutrophil to lymphocyte ratio (NLR)



2.5.5s C-Reactive protein (CRP)



2.5.6s Albumin

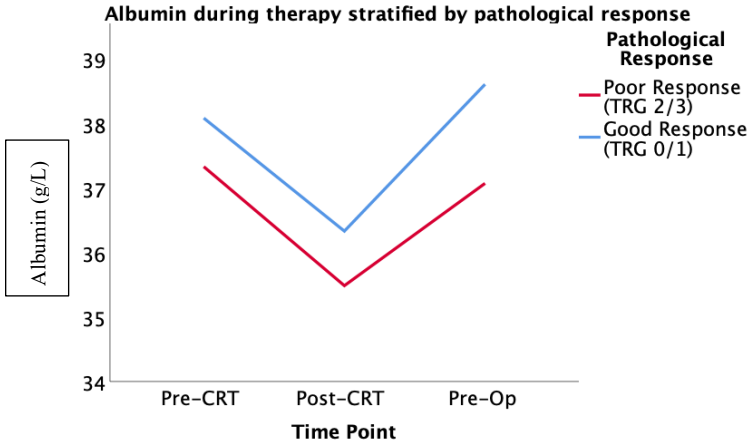


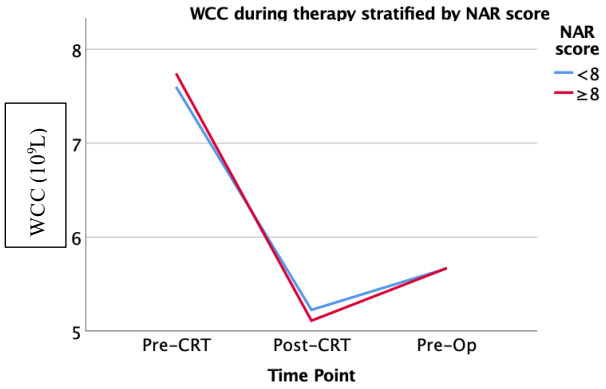
Table 2.9s. Median values of white cell count, its constituents, NLR, C-Reactive protein and albumin over time P values Mann-Whitney U test comparing NAR score an indicator of prognosis (NAR<8 good prognosis and NAR ≥8 intermediate / poor prognosis) at each time point.

	Timepoint	**NAR Score**		**p value**
		NAR <8	**NAR ≥8**	
WCC (10^9/L)	Pre-CRT	6.98	7.75	0.238
	Post-CRT	5.66	4.94	0.182
	Pre-Op	5.65	5.50	0.178
Neutrophils (10^9/L)	Pre-CRT	4.08	5.00	0.163
	Post-CRT	3.48	3.24	0.088
	Pre-Op	3.80	3.80	0.119
Lymphocytes (10^9/L)	Pre-CRT	1.75	1.70	0.859
	Post-CRT	0.52	0.68	0.428
	Pre-Op	1.05	0.85	0.920
NLR	Pre-CRT	2.84	2.86	0.060
	Post-CRT	5.94	4.99	0.073
	Pre-Op	3.74	4.29	0.100
CRP (mg/L)	Pre-CRT	3.0	3.8	0.548
	Post-CRT	2.1	3.0	0.437
	Pre-Op	1.7	3.4	0.199
Albumin (g/L)	Pre-CRT	38	37	0.148
	Post-CRT	36	36	0.209
	Pre-Op	37	37	0.013

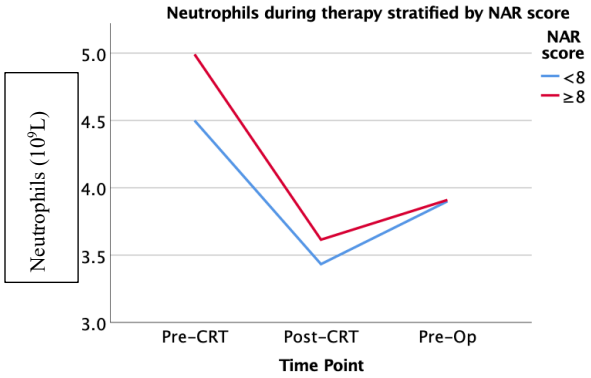
CRT chemoradiotherapy; WBC total white cell count; NLR neutrophil to lymphocyte ratio; CRP C-reactive protein.

Figures 2.6s Median values of serum markers prior to and following neoadjuvant therapy stratified by neoadjuvant rectal (NAR) score. < 8 indicates good prognosis and ≥ 8 indicates moderate / poor prognosis.

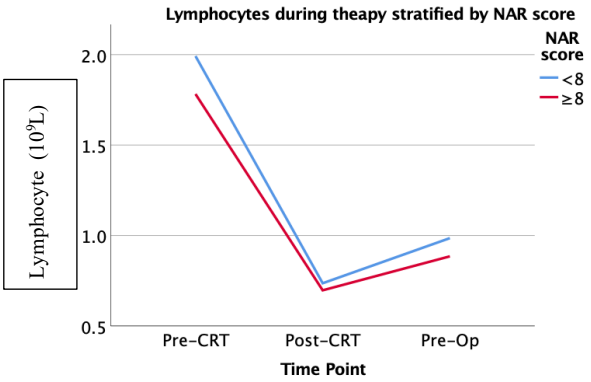
2.6.1s White cell count (WCC)



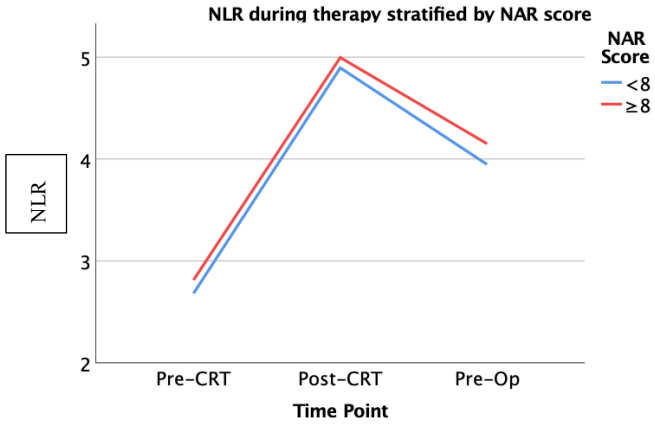
2.6.2s Neutrophil count



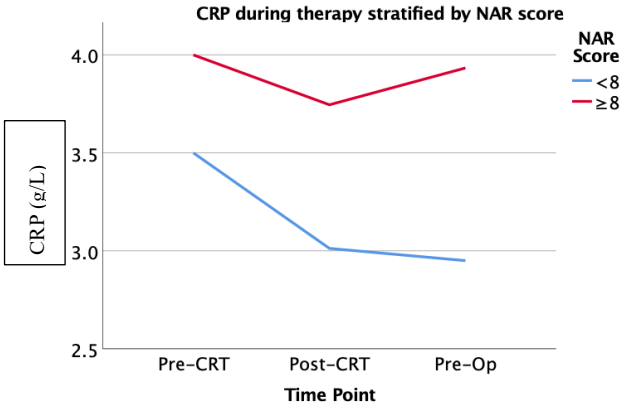
2.6.3s Lymphocyte count



2.6.4s Neutrophil to lymphocyte ratio (NLR)



2.6.5s C-Reactive protein (CRP)



2.6.6s Albumin

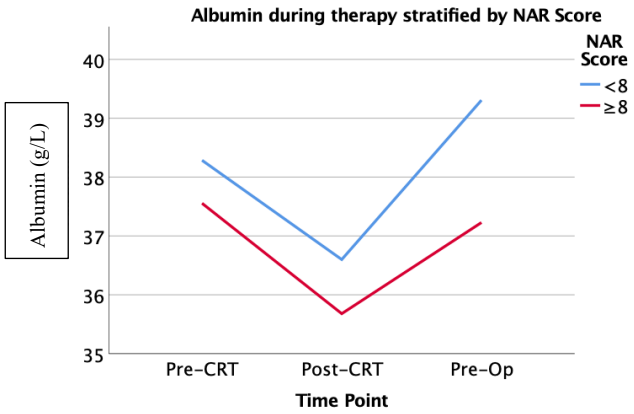


Table 2.10s. Median values of white cell count, its constituents, NLR, C-Reactive protein and albumin over time P values Mann-Whitney U test comparing the presence of pathological complete response at each time point.

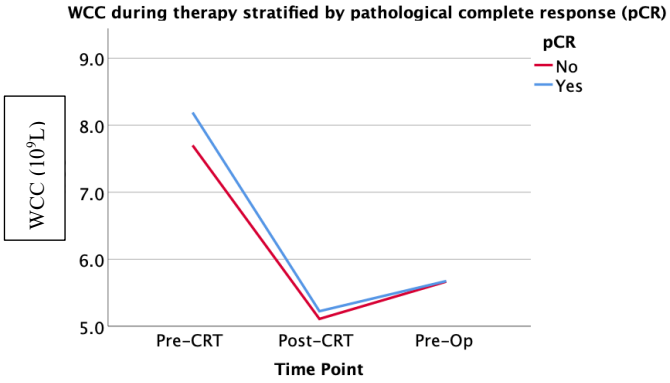
	Timepoint	**pCR**		**p value**
		Yes	**No**	
WCC (10^9/L)	Pre-CRT	7.30	7.64	0.778
	Post-CRT	5.10	5.09	0.671
	Pre-Op	5.80	5.50	0.235
Neutrophils (10^9/L)	Pre-CRT	4.10	4.99	0.741
	Post-CRT	3.27	2.34	0.887
	Pre-Op	4.10	3.75	0.403
Lymphocytes (10^9/L)	Pre-CRT	1.75	1.70	0.160
	Post-CRT	0.51	0.68	0.236
	Pre-Op	1.05	0.90	0.174
NLR	Pre-CRT	2.84	2.86	0.439
	Post-CRT	6.51	4.91	0.502
	Pre-Op	3.99	4.13	0.967
CRP (mg/L)	Pre-CRT	3.00	3.75	0.856
	Post-CRT	3.00	3.00	0.541
	Pre-Op	1.85	3.05	0.219

Albumin (µg/L)	Pre-CRT	38	37	0.052
	Post-CRT	35	36	0.018
	Pre-Op	37	37	0.052

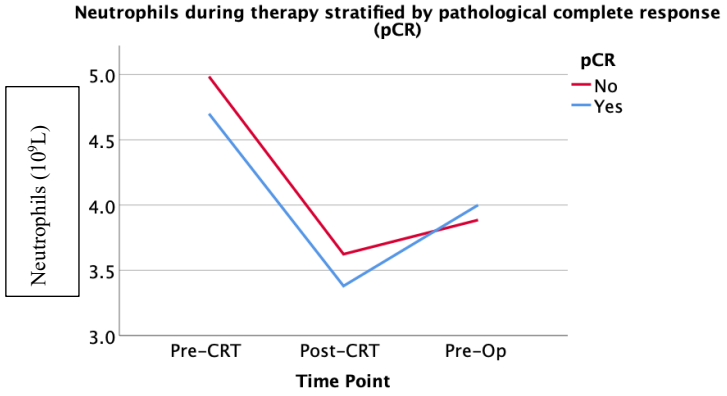
CRT chemoradiotherapy; WBC total white cell count; NLR neutrophil to lymphocyte ratio; Hb Haemoglobin; CEA carcinoembryonic antigen; CRP C-reactive protein.

Figures 2.7s Median values of serum markers prior to and following neoadjuvant therapy stratified by pathological complete response (pCR)

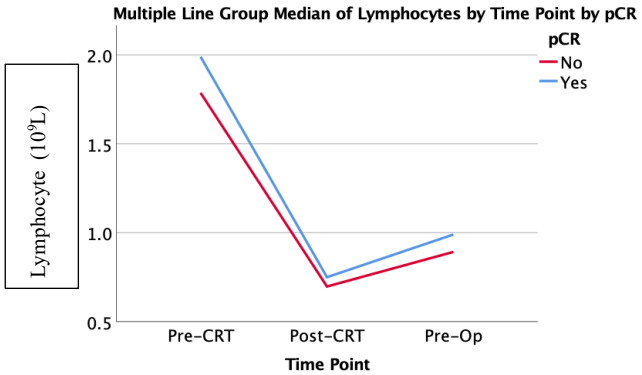
2.7.1s White cell count (WCC)



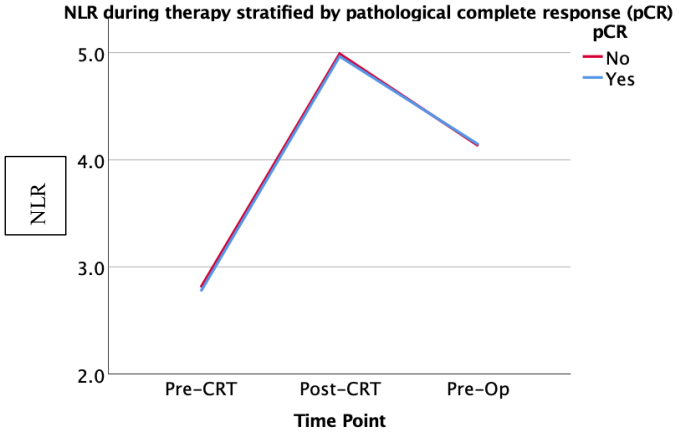
2.7.2s Neutrophil count



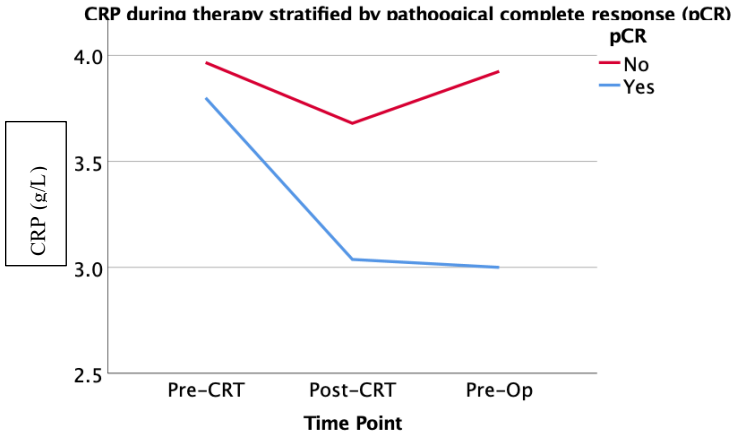
2.7.3s Lymphocyte count



2.7.4s Neutrophil to lymphocyte ratio (NLR)



2.7.5s C-Reactive protein (CRP)



2.7.6s Albumin

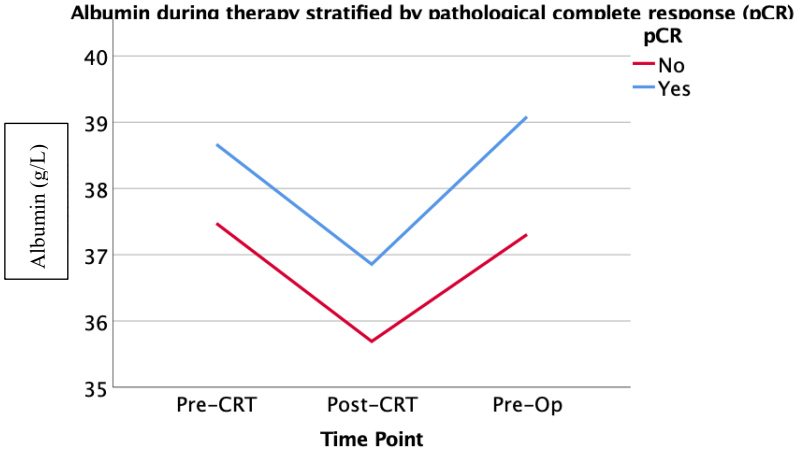


Table 2.11s. Relationship between changes in NLR (<3 Low and ≥ 3 high), CRP, CEA, mGPS and pathological tumour regression. P values from Chi square testing. Numbers in parenthesis indicate percentages.

			Response (%)		P value
			Good (TRG 0/1)	Poor (TRG 2/3)	
NLR Baseline to Post-CRT	Low - Low	32 (15)	15 (20)	17 (12.8)	0.635
	Low - High	88 (42)	31 (40)	57 (43)	
	High - High	87 (41)	30 (39)	57 (43)	
	High - Low	3 (1)	1 (1)	2 (2)	
NLR Baseline to Pre-Op	Low - Low	51 (24)	21 (26)	30 (22)	0.861
	Low - High	74 (34)	29 (36)	45 (34)	
	High - High	72 (25)	25 (31)	47 (35)	
	High - Low	18 (8)	6 (7)	12 (9)	
CRP Baseline to Post-CRT	Low - Low	74 (70)	25 (74)	49 (68)	0.171
	Low - High	15 (14)	7 (21)	8 (11)	
	High - High	5 (5)	1 (3)	4 (6)	
	High - Low	12 (11)	1 (3)	11 (15)	
CRP Baseline to Pre-Op	Low - Low	73 (80)	28 (85)	45 (78)	0.274
	Low - High	5 (6)	3 (9)	2 (3)	
	High - High	8 (9)	1 (3)	7 (12)	
	High - Low	5 (6)	1 (3)	4 (7)	
CRP Baseline to Post-CRT	Low Low or Low High	89 (84)	32 (94)	57 (79)	0.050
	High High or High Low	17 (16)	2 (6)	15 (21)	
CRP Baseline to Pre-Op	Low Low or Low High	78 (86)	31 (94)	47 (81)	0.091
	High High or High Low	13 (14)	2 (6)	11 (19)	
CEA Baseline to Post-CRT	Low - Low	63 (58)	33 (72)	30 (48)	0.071
	Low - High	2 (2)	0	2 (3)	
	High - High	34 (31)	10 (22)	24 (38)	

	High - Low	10 (9)	3 (7)	7 (11)	
mGPS Baseline to Post-CRT	Static	84 (80)	25 (74)	59 (82)	0.564
	Fall	6 (6)	2 (6)	4 (6)	
	Rise	16 (15)	7 (21)	9 (13)	
mGPS Baseline to Pre-Op	Static	76 (81)	28 (82)	48 (80)	0.768
	Fall	11 (12)	3 (9)	8 (13)	
	Rise	7 (7)	3 (9)	4 (7)	
mGPS 0/1/2 Baseline to Post-CRT	Static - Low	74 (70)	25 (74)	49 (68)	0.120
	Fall	6 (6)	2 (6)	4 (6)	
	Rise	16(15)	7 (21)	9 (13)	
	Static - High	10 (9)	0	10 (14)	
mGPS 0 /1 /2 Baseline to Pre-Op	Static - Low	73 (78)	28 (82)	45 (75)	0.500
	Fall	11 (12)	3 (9)	8 (13)	
	Rise	7 (7)	3 (9)	4 (7)	
	Static - High	3 (3)	0	3 (5)	
mGPS 0 / 1-2 Baseline to Post-CRT	Low - Low	74 (70)	25 (74)	49 (68)	0.171
	Low - High	15 (14)	7 (21)	8 (11)	
	High - High	12 (11)	1 (3)	11 (15)	
	High - Low	5 (5)	1 (3)	4 (6)	
mGPS 0 / 1-2 Baseline to Pre-Op	Low - Low	73 (78)	28 (82)	45 (75)	0.321
	Low – High	5 (5)	3 (9)	2 (3)	
	High - High	6 (6)	1 (3)	5 (8)	
	High - Low	10 (11)	2 (6)	8 (13)	

Table 2.12s Relationship between changes in NLR, CRP, CEA, mGPS and NAR score. P values from Chi square testing. Numbers in parenthesis indicate percentages.

			NAR (%)		P value
			<8	≥8	
NLR Baseline to Post-CRT	Low - Low	33 (15)	11 (22)	22(13)	0.296
	Low - High	94 (43)	18 (36)	76 (44)	
	High - High	91 (41)	21 (42)	90 (41)	
	High - Low	3 (1)	0	3 (2)	
NLR Baseline to Pre-Op	Low - Low	55 (24)	14 (41)	41 (24)	0.928
	Low - High	77 (34)	17 (33)	60 (35)	
	High - High	75 (33)	16 (31)	59 (34)	
	High - Low	19 (8)	5 (10)	14 (8)	
CRP Baseline to Post-CRT	Low - Low	77 (71)	16 (76)	61 (69)	0.431
	Low - High	15 (14)	4 (19)	11 (13)	
	High - High	5 (5)	0	5 (6)	
	High - Low	12 (11)	1 (5)	11 (13)	
CRP Baseline to Pre-Op	Low - Low	74 (80)	17 (90)	57 (78)	0.190
	Low - High	5 (5)	2 (10)	3 (4)	
	High - High	8 (9)	0	8 (11)	
	High - Low	5 (5)	0	5 (7)	
CRP Baseline to Post-CRT	Low Low or Low High	92 (84)	20 (95)	72 (82)	0.128
	High High or High Low	17 (16)	1 (5)	16 (18)	
CRP Baseline to Pre-Op	Low Low or Low High	79 (86)	19 (100)	60 (82)	0.047
	High High or High Low	13 (14)	0 (0)	13 (18)	
CEA Baseline to Post-CRT	Low - Low	65 (58)	25 (81)	40 (49)	0.026
	Low - High	2 (2)	0	2 (3)	
	High - High	35 (31)	5 (16)	10 (37)	
	High - Low	10 (9)	1 (3)	9 (11)	
mGPS	Static	87 (80)	16 (76)	71 (81)	0.229

Baseline to Post-CRT	Fall	6 (6)	0	6 (7)	
	Rise	16 (15)	5 (24)	11 (13)	
mGPS Baseline to Pre Op	Static	77 (81)	17 (90)	60 (79)	0.194
	Fall	11 (12)	0	11 (15)	
	Rise	7 (7)	2 (10)	5 (7)	
mGPS 0/1/2 Baseline to Post-CRT	Static - Low	77 (71)	16 (76)	61 (70)	0.144
	Fall	6 (6)	0	6 (7)	
	Rise	16 (15)	5 (24)	11 (13)	
	Static - High	10 (9)	0	10 (9)	
mGPS 0/1/2 Baseline to Pre-Op	Static - Low	74 (78)	17 (90)	57 (75)	0.238
	Fall	11 (12)	0	11 (15)	
	Rise	7 (7)	2 (10)	5 (7)	
	Static - High	3 (3)	0	3 (4)	
mGPS 0/1-2 Baseline to Post-CRT	Low - Low	77 (71)	16 (76)	61 (69)	0.431
	Low - High	15 (14)	4 (19)	11 (13)	
	High - High	12 (11)	1 (5)	11 (13)	
	High - Low	5 (5	0	5 (6)	
mGPS 0/1-2 Baseline to Pre-Op	Low - Low	74 (78)	17 (90)	57 (75)	0.129
	Low – High	5 (5)	2 (11)	3 (4)	
	High - High	6 (6)	0	6 (8)	
	High - Low	10 (11)	0	10 (13)	

Table 2.13s. Relationship between changes in NLR, CRP, CEA, mGPS and pathological complete response. P values from Chi square testing. Numbers in parenthesis indicate percentages.

			pCR (%)		P value
			Yes	No	
NLR Baseline to Post-CRT	Low - Low	33 (15)	10 (23)	23 (13)	0.266
	Low - High	94 (43)	15 (34)	79 (45)	
	High - High	91 (41)	19 (43)	72 (41)	
	High - Low	3 (1)	0	3 (2)	
NLR Baseline to Pre-Op	Low - Low	55 (24)	11 (24)	44 (24)	0.999
	Low - High	77 (34)	15 (33)	62 (34)	
	High - High	75 (33)	15 (33)	60 (33)	
	High - Low	10 (8)	4 (9)	15 (8)	
CRP Baseline to Post-CRT	Low - Low	77 (71)	13 (72)	64 (70)	0.440
	Low - High	15 (14)	4 (22)	11 (12)	
	High - High	5 (5)	0	5 (6)	
	High - Low	12 (11)	1 (6)	11 (12)	
CRP Baseline to Pre-Op	Low - Low	64 (80)	16 (94)	58 (77)	0.329
	Low - High	5 (5)	1 (6)	4 (5)	
	High - High	8 (9)	0	8 (11)	
	High - Low	5 (5)	0	5 (7)	
CRP Baseline to Post-CRT	Low Low or Low High	92 (84)	17 (94)	75 (82)	0.199
	High High or High Low	17 (16)	1 (6)	16 (18)	
CRP Baseline to Pre-Op	Low Low or Low High	79 (86)	17 (100)	62 (83)	0.064
	High High or High Low	13 (14)	0 (0)	13 (17)	
CEA Baseline to Post-CRT	Low - Low	65 (58)	22 (85)	43 (50)	0.019
	Low - High	2 (2)	0	2 (2)	
	High - High	35 (31)	3 (11)	32 (37)	
	High - Low	10 (9)	1 (4)	9 (10)	

mGPS Baseline to Post-CRT	Static	87 (80)	13 (72)	74 (81)	0.145
	Fall	6 (6)	0	6 (7)	
	Rise	16 (15)	5 (28)	11 (12)	
mGPS Baseline to Pre Op	Static	77 (81)	16 (94)	61 (78)	0.235
	Fall	11 (12)	0	11 (14)	
	Rise	7 (7)	1 (6)	6 (8)	
mGPS 0/1/2 Baseline to Post-CRT	Static - Low	77 (71)	13 (72)	64 (70)	0.128
	Fall	6 (6)	0	6 (7)	
	Rise	16 (15)	5 (28)	11 (12)	
	Static - High	10 (9)	0	10 (11)	
mGPS 0 /1 /2 Baseline to Pre-Op	Static - Low	74 (78)	16 (94)	58 (74)	0.282
	Fall	11 (12)	0	11 (14)	
	Rise	7 (7)	1 (6)	6 (8)	
	Static - High	3 (3)	0	3 (4)	
mGPS 0 / 1-2 Baseline to Post-CRT	Low - Low	77 (71)	13 (72)	64 (70)	0.440
	Low - High	15 (14)	4 (22)	11 (12)	
	High - High	12 (11)	1 (6)	11 (12)	
	High - Low	5 (5)	0	5 (6)	
mGPS 0 / -2 Baseline to Pre-Op	Low - Low	74 (78)	16 (94)	58 (74)	0.240
	Low – High	5 (5)	1 (6)	4 (5)	
	High - High	6 (6)	0	6 (8)	
	High - Low	10 (11)	0	10 (13)	

Appendix II

An investigation into the potential immune priming effect of radiotherapy on the tumour microenvironment in rectal cancer

Running title: Immune priming in Rectal Cancer Study

Protocol Version: Version 3

Date: 29/03/19

REC Reference Number: 18WS0003

Sponsor's Protocol Number: GN17ON712

Sponsor: NHS Greater Glasgow & Clyde>

Funder: Beatson Cancer Charity

Amendment number	Date	Protocol version
1	24/01/18	V2
2	29/01/18	V3

This study will be performed according to the Research Governance Framework for Health and Community Care (Second edition, 2006) and WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI Ethical Principles for Medical Research Involving Human Subjects 1964 (as amended).

CONTACTS

Chief Investigator

Dr Campbell Roxburgh

Senior Clinical Lecturer and Honorary Consultant Colorectal Surgeon

Room 2.60, New Lister Building, Glasgow Royal Infirmary, G31 2ER

Tel: 0141 201 8621

E-mail: Campbell.Roxburgh@glasgow.ac.uk

Co-investigator

Dr Harikrishnan Nair

Clinical Research Fellow, General Surgery, University of Glasgow.

Room 2.56, New Lister Building, Glasgow Royal Infirmary, G31 2ER

Tel: 0141 201 8674

E-mail: Harikrishnan.Nair@glasgow.ac.uk

Funding Body – see section 10

Beatson Cancer Charity

1053 Great Wester Road, G12 0YN

STUDY SYNOPSIS

Title of Study:	An investigation into the potential immune priming effect of radiotherapy on the tumour microenvironment in rectal cancer
Study Centre:	Beatson Cancer Centre/ Glasgow Royal Infirmary
Duration of Study:	Until recruitment complete – up to 4 years
Primary Objective:	To evaluate whether different forms of external beam radiotherapy (e.g. long course vs short course regimens) influence immune responses?
Secondary Objective:	(a) To investigate when such immune responses peak and for what duration they persist? (b) Whether molecular make-up of the tumour has any effect on degree of priming response? (c) Whether degree of immunogenicity plays a role in tumour downstaging responses
Primary Endpoint:	Evidence of T cell infiltrates present in tumour biopsies during treatment
Rationale:	Immunotherapies have revolutionized cancer treatment in many tumour types. However, these drugs are not effective in all cancers. Indicators that immunotherapy will be effective include evidence of an 'immune active' tumour microenvironment. This includes high numbers of tumour infiltrating T cells, evidence of antigen presentation (MHC-1 expression) and expression of immune checkpoint molecules (PD-1/ PDL-1). The majority of colorectal cancers demonstrate low numbers of tumour infiltrating T cells and expression of immune checkpoint molecules. Consequently, checkpoint blockade immunotherapy is limited to one subtype comprising 15% of all disease and 5% of metastatic

	tumours: the mismatch repair deficient (dMMR) subtype. Strategies to enhance tumour immunogenicity may expand the role of immunotherapy beyond the dMMR subtype. In this pilot study, we evaluate the intra-tumoral immune effects of radiotherapy and chemotherapy using serial biopsies. By defining the patterns of immune response during treatment we may be able to plan trials of combinations with immunotherapy in rectal cancer.
Methodology:	Prospective pilot study Patients treated with radiotherapy for rectal cancer at the Beatson West of Scotland Cancer Centre will be recruited. Two to four biopsies will be performed by procto-sigmoidoscopy at the time patients attend for clinical assessments. Biopsies will be performed at baseline, 2-3 weeks, 6 weeks and at 12 weeks during and after radiotherapy. Blood samples will be collected at similar time points. Samples will be assessed for: - Molecular subtyping (151 gene panel assessment) - Immune cell infiltrates (Immunohistochemistry) - Transcriptional alterations (Nanostring gene expression) - Circulating cytokines/ chemokines
Sample Size:	30
Screening:	Patients with rectal cancer scheduled to receive radiotherapy will be identified through the colorectal multidisciplinary team meeting. They will be approached in clinic by a surgeon on oncologist involved in the study.
Registration/Randomisation:	Any recruits will be given a unique study ID and data stored in anonymised form on secure password protected University of Glasgow computers within the Academic Unit of surgery, Glasgow Royal Infirmary. No randomisation required.

Main Inclusion Criteria:	• - Over 18 years of age • - Patients with known rectal cancer (adenocarcinoma) which is due to be treated with radiotherapy • - Capacity to provide informed consent • - Willingness to allow additional tumour biopsies to be performed at clinical assessments
Main Exclusion Criteria:	• - Under 18 years of age • - Patients with bleeding disorders • - Patients prescribed anticoagulants in whom a bleeding risk would be present (warfarin, dalteparin, apixiban) • - Patients with tumours at or below the dentate line of the anal canal
Product, Dose, Modes of Administration:	N/A
Duration of Treatment:	Involvement in the study will last 4 weeks
Statistical Analysis:	

Title

An investigation into the potential immune priming effect of radiotherapy on the tumour microenvironment in rectal cancer

Introduction

Rationale

Immunotherapies have revolutionized cancer treatment in many tumour types. However, these drugs are not effective in all cancers. Indicators that immunotherapy will be effective include evidence of an 'immune active' tumour microenvironment. This includes high numbers of tumour infiltrating T cells, evidence of antigen presentation (MHC-1 expression) and expression of immune checkpoint molecules (PD-1/ PDL-1). The majority of colorectal cancers demonstrate low numbers of tumour infiltrating T cells and expression of immune checkpoint molecules. Consequently, checkpoint blockade immunotherapy is limited to one subtype comprising 15% of all disease and 5% of metastatic tumours: the mismatch repair deficient (dMMR) subtype. Strategies to enhance tumour immunogenicity may expand the role of immunotherapy beyond the dMMR subtype. In this pilot study, we evaluate the intra-tumoral immune effects of radiotherapy and chemotherapy using serial biopsies. By defining the patterns of immune response during treatment we may be able to plan trials of combinations with immunotherapy in rectal cancer.

Background information including literature review

Colorectal cancer (CRC) is the second leading cause of death in the UK with 41,000 new cases diagnosed per year and 16,000 deaths. Rectal cancer accounts for 1/3 of disease. Additional treatments are therefore required to improve survival rates. Rectal cancer management differs from that of colon cancer in that radiotherapy (RT) is more commonly utilized in the neoadjuvant and palliative settings. Radiotherapy is a DNA damaging treatment which leads to tumour cell death. However, RT is also capable of inducing several immunological effects due to immunogenic cell death with release of tumour antigens.

Immunotherapies are novel treatments, effective in various tumour types including melanoma, where checkpoint blockade of CTLA-4 and PD-1 T-cell inhibitory molecules improves survival (1, 2). Tumours thought to be responsive to checkpoint blockade immunotherapy include those with high mutational load and neoantigen burden (3), or evidence of an immune active tumour microenvironment (an abundance of T cell infiltrates or increased MHC-1 expression) (4, 5). PD-1/PD-L1 expression and presence of T cells within the tumour microenvironment are thought to represent the most useful current biomarkers that anti PD-1 therapy may be effective.

The use of immunotherapy in CRC is currently very limited. Most CRCs have low numbers of T cells present within the tumour microenvironment and PD-1 is poorly expressed in CRC (13%) compared with melanomas (53%) (6). CRCs that exhibit the defective mismatch repair (dMMR) molecular subtype demonstrate enhanced immunogenicity/ T cell infiltrates within the tumour's microenvironment which are thought to be responding to neo-antigens produced by the higher mutation rate (7). Encouragingly, phase II clinical trials in dMMR CRC report improved progression free survival and overall survival with anti-PD-1 immunotherapy (8, 9). dMMR tumours make up only 15% of CRCs, very few of which develop metastases, consequently anti-PD-1 therapy applies to a tiny subset of all CRCs. Strategies to enhance immunogenicity in other CRC subtypes may allow a broader use of immunotherapy in CRC. Such treatments may synergize with immunotherapies, to enhance response and improve outcomes.

Importantly, radiotherapy is capable of inducing T cell responses responding to antigens released by tumour cell death (10). Previous studies have demonstrated that chemoradiation (CRT) to rectal tumours results in CD4+/CD8+ T cells in resected specimens when compared to pre-treatment biopsies (11, 12). However, little is known regarding the temporal changes in immunological profiles during and after treatment. It is important to study these immunological effects of RT to rectal tumours in order to plan potential trials of immunotherapies in combination with RT for rectal cancer. Such a strategy may expand the role of immunotherapies beyond the dMMR subgroup. Rectal tumours are accessible at proctoscopy performed in the clinic or endoscopy suite with minimal patient discomfort and represent the ideal tumour type to perform such work. In patients that consent to be involved in this study, we will analyse immune cell infiltrates and expression of immunological proteins on tumour tissue before, during and after different types of radiotherapy in rectal cancer. The main types of radiotherapy used in rectal cancer are short course (high dose per fraction given over short period of 5 days) and long course radiotherapy (lower dose per fraction given over 28 days in combination with chemotherapy (5-FU or capecitabine). Although short course treatment provides a relatively high dose in a short time period the cumulative dose is higher with long course treatment and therefore the degree of priming of the immune response and its duration may differ between therapies.

Potential risk and benefits

Potential Risk: Biopsies are routinely performed in rectal cancer without any significant risk. However, in a very small proportion of patients particularly those on anticoagulants, a small amount of bleeding can occur. In some patients with very low tumours, significant discomfort can be experienced during the proctosigmoidoscopy, but we will aim to avoid recruiting patients with very low tumours (i.e. those at the dentate line). We will be using a patient questionnaire (Appendix IV) to determine the acceptability of serial endoscopic examinations.

Prior experience of intervention

Previous work has demonstrated it is possible to reliably sample rectal cancers during different cancer treatments using proctosigmoidoscopy and biopsy to provide details of tissue biomarkers including immunological profiles during chemotherapy (13, 14). We aim to perform similar analyses for the purposes of this study.

Study Hypothesis

Treatment with radiotherapy or chemotherapy is capable of inducing potentially favourable immunological effects. We hypothesise that there will be differences in the peak and duration of these effects between different forms of radiotherapy (short course and long course).

Aim/Primary and Secondary Objectives

We aim to perform a pilot study to evaluate immune cell profiles and immunological protein expression in tumour biopsies using immunohistochemistry. We will perform a colorectal cancer gene panel assessment at baseline to provide an assessment of CRC molecular features.

Primary Objective

To evaluate whether different forms of external beam radiotherapy (e.g. long course vs short course regimens) influence immune responses?

Secondary Objectives

(a) To investigate when such immune responses peak and for what duration they persist?

(b) Whether molecular make-up of the tumour has any effect on degree of priming response?

(c) Whether degree of immunogenicity plays a role in tumour downstaging responses

(d) Assess patient acceptability to repeated endoscopic assessments

Research Questions

RQ1: Can immune cell responses and expression of MHC-1, PD-1 and PD-L1 be measured on sequential tumour biopsies to provide an assessment of patterns of immune priming in response to radiotherapy?

RQ2: Does the immune response generated (intensity, peak and duration) by short course radiotherapy differ in comparison to long course chemoradiotherapy.

RQ3: Is there a common time point for each type of treatment when such immune responses peak and for what duration they persist?

RQ4: Does the molecular make-up and mutational profile of the tumour have any effect on degree of priming response?

RQ4: Does the degree of immunogenicity play any role in the degree of tumour response or downstaging with radiotherapy.

RQ5: Are there gene signatures (measured using Nanostring nCounter gene expression analysis) that can be applied to stratify a subset of patients by degree of immunogenicity present at baseline and during treatment

RQ6: Are serial endoscopic assessments acceptable to patients.

3. Study Design

After appropriate ethical approval, a pilot study will be performed in 30 patients treated with radiotherapy for rectal cancer at the Beatson West of Scotland Cancer Centre. This study will be performed according to the Research Governance Framework for Health and Community Care (Second edition, 2006).

3.1 Study Population

Thirty patients meeting the eligibility criteria will be approached. We hope to recruit approximately 15 patients who are receiving short course radiotherapy and 15 scheduled to receive long course chemo-radiotherapy. Patients will be identified through the colorectal cancer multidisciplinary team meeting and will be approached at Surgical or Oncology clinics at Glasgow Royal Infirmary, Stobhill Hospital or The Beatson West of Scotland Cancer Centre. Each potential participant will be given a Patient Information Sheet and at a follow up visit invited to provide informed consent in order to participate in the study. The study will involve volunteering to allow additional biopsies and blood samples to be taken for research purposes at 4 time points as they undergo treatment with radiotherapy for rectal cancer. Two to four biopsies will be performed by procto-sigmoidoscopy at the time patients attend for clinical assessments. These will be performed in the outpatient clinic or at the same time the patient attends for other assessments if this happens to fall at a convenient time point. For example, some patients may attend endoscopy units for a post-treatment assessment of response. Procto-sigmoidoscopy is a commonly performed assessment in the outpatient clinic and plays a role in the assessment of tumour response. All patients recruited to the study will be aware of what is likely to be involved in this test due to the fact that initial diagnosis of the rectal cancer will have involved some form of endoscopic assessment. Biopsies from the rectum above the dentate line should not cause discomfort.

3.2 Inclusion criteria

- Over 18 years of age
- Patients with known rectal cancer (adenocarcinoma) which is due to be treated with radiotherapy
- Capacity to provide informed consent
- Willingness to allow additional tumour biopsies to be performed at clinical assessments
- The ability to understand simple written and verbal English

3.3 Exclusion criteria

- Under 18 years of age
- Patients with bleeding disorders
- Patients prescribed anticoagulants in whom a bleeding risk would be present (warfarin, dalteparin, apixiban)
- Patients with tumours at or below the dentate line of the anal canal

3.4 Identification of participants and consent

Patients will be identified through the colorectal cancer multidisciplinary team meeting and will be approached at Surgical or Oncology clinics at Glasgow Royal Infirmary or The Beatson West of Scotland Cancer Centre. Each potential participant will be given a Patient Information Sheet and at a follow up visit invited to provide informed consent in order to participate in the study. Care will be taken to ensure potential participants are aware of the requirements of the study protocol in that 1-2 of these proctosigmoidoscopy assessments and the biopsies taken do not constitute standard management for their condition. Patients will also be made aware that participation in this research study is entirely voluntary and should they choose not to be involved or withdraw at any point this will not affect their cancer treatment. Patients who lack capacity will not be approached.

3.5 Withdrawal of subjects

Patients who choose to withdraw can do so at any point without requirement to provide an explanation. If they wish any data collected up to that point to be excluded from the study's analysis all data collected to that date can be deleted. The study will aim to recruit until we reach 30 patients. If a patient develops complications related to their cancer treatment or becomes unwell in another way, preventing them completing their radiotherapy treatment as per local protocols, then it is likely we would withdraw these patients from the study. This is because they would not receive the standard radiotherapy doses at the desired time points required to interpret the results from on-treatment biopsies.

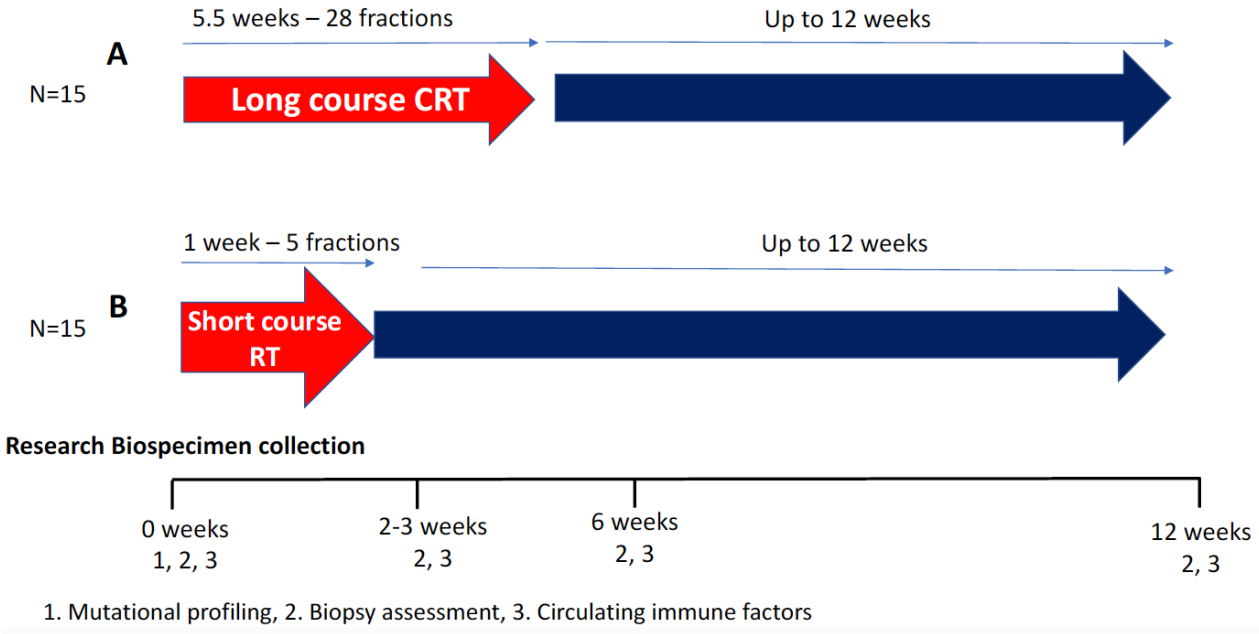
4 Study Outcome Measures

Measurements/ Outcomes

We will ask patients undergoing radiotherapy treatment for permission to perform serial biopsies prior to, during and after treatment with radiotherapy (at 0, 3, 6 and 12 weeks in long course regimens, 0, 2, 6 and 12 weeks in short course regimens). A blood sample will also be performed at each timepoint. On each occasion, the biopsies will be placed in formalin and transferred that day by a member of the research team to Glasgow Biorepository, where they will be cold stored prior to tissue processing. Once tissue has been sectioned and approved by the pathologist for research use, the tissue will be transferred to Dr Joanne Edwards lab at the Wolfson Wohl Cancer Research Centre, Glasgow.

Biospecimen collection time points:

[Grab your reader's attention with a great quote from the document or use this space to emphasize a key point. To place this text box anywhere on the page, just drag it.]



1. Molecular subtyping and assessment of mutations present.

Samples will undergo tumour normal pair sequencing using a comprehensive cancer gene panel of 151 genes. This includes the 30 most common driver mutations in colorectal cancer and will provide a signal for mutational load. Data obtained will be analysed using a systems biology approach and bioinformatic readouts to be utilised include simple somatic mutations, copy number alterations and mutational signature. This work will be performed in collaboration with the David Chang, Peter Bailey and sequencing team at the Wolfson Wohl Cancer Research Centre, University of Glasgow.

Time Points for analysis: 0 weeks Sample collection: FFPE and whole blood (frozen)

2. Immune cell infiltrates in tumour tissue

IHC will be performed for the following antibodies:

Measures of T cell responses	CD3, CD8 and FOXP3
Measures of myeloid responses	CD11b/Gr-1, CD14, CD68
Measures of increased antigen presentation	MHC-1, MHC-II
Immune checkpoint expression	PD-1, PD-L1, CTLA-4.

This work will be performed in collaboration with Dr Joanne Edwards' Lab at the Wolfson Wohl Cancer Research Centre, Glasgow. We have experience quantifying these markers on tumour biopsies, using a point count method. This can be done manually or using an automated system.

Time Points for analysis: At 0 weeks, 2-3 weeks, 6 weeks and 12 weeks. Sample collection: FFPE

3. Transcriptional alterations

NanoString nCounter Analysis system will be used to allow analysis of RNA extracted from formalin-fixed paraffin embedded (FFPE) cancer tissue curls. NanoString nCounter gene expression analysis will be performed for the nCounter GX Human Kinase array (519 kinase genes) and nCounter Human Cancer reference array (230 cancer genes). These arrays will allow temporal changes in immune/ inflammatory signatures to be evaluated before, during and after treatment. Data obtained will be analysed using a systems biology approach. This work will be performed in subset who exhibit strong T cell responses (N=10). This work will be performed in collaboration with the David Chang and Joanne Edwards groups.

Time Points for analysis: At 0 weeks, 2-3 weeks, 6 weeks and 12 weeks. Sample collection: FFPE

4. Circulating immunological parameters

Circulating immunological parameters will also be evaluated to assess for temporal changes in the systemic circulation. This work will be performed by the Biochemistry department and in collaboration with Joanne Edwards group.

Measures of interest include:

Routinely assessed parameters:	Differential white cell count
	C-reactive protein and albumin
Circulating cytokines:	e.g. ELISAs for IL-1, Il-6, TNF-alpha, IL-10
Flow cytometry:	Circulating T cell populations
	Circulating myeloid populations

Time Points for analysis: At 0 weeks, 2-3 weeks, 6 weeks and 12 weeks. Sample collection: blood.

5. Circulating cell free DNA (cfNDA)

Analysis of circulating cell-free DNA (cfDNA) from serum or plasma samples will allow for a 'snapshot' of the total mutational landscape at each sampling time-point. The use of such liquid biopsies has been previously shown to identify the presence of occult metastases (29).

Time Points for analysis: At 0 weeks, 2-3 weeks, 6 weeks and 12 weeks. Sample collection: blood.

Sample collection/ storage

After biopsies are performed, they will be placed in formalin and the container will be labelled with a unique anonymized Study ID by Glasgow Biorepository for processing before transfer to the Wolfson Wohl Cancer Research Building. Blood samples will be similarly labelled and transferred for storage at -85 ºC . Blood sample for cfDNA will be couriered at

5. Assessment of Safety

Although it is not anticipated that these additional biopsies and blood samples taken during treatment will result in any adverse events, we aim to ensure these procedures are well tolerated and do not result in additional patient concern.

6. Statistics and Data Analysis

The cohort size of 30 has been chosen as this is a signal finding pilot study. If a strong signal was demonstrated we may consider expanding the study with appropriate additional ethical approval and funding applications as required. For example, if we consider evidence of an immune priming response as the presence of mod-high grade T cell infiltrates in >50% of patients and baseline responses are present in 20-25%, then in order to detect a significant difference in priming responses (>25%) at a set time point we will require 28 patients in each group (short vs long course). At the outset, we will plan to recruit 30 patients (15+15).

Patient identifiable information including the consent form will be linked to the Study ID and stored in a locked study file within the Academic Unit of Surgery on Level 2, New Lister Building, Glasgow Royal Infirmary. All electronic data will be stored on University servers or on an encrypted hard drive. Access will be by named study researchers including Dr Campbell Roxburgh

A letter will be sent to the patients GP to inform them of study participation.

7. Study Closure

Each patient will be involved in the research study for 12 weeks in total. The study will end when the study team agrees that our recruitment target has been met (N=30). However, the study will not continue beyond 4 years and if recruitment is so poor that completion cannot be completed within this time frame then the study would be discontinued.

8. Protocol Amendments

Any change in the study protocol will require an amendment. Any proposed protocol amendments will be initiated by the CI, Campbell Roxburgh following discussion with the study team and any required amendment forms will be submitted to the regulatory authority, ethics committee and sponsor. The study team will liaise with study sponsor to determine whether an amendment is non-substantial or substantial. All amended versions of the protocol will be signed by the CI and Sponsor representative. Before the amended protocol can be implemented favourable opinion/approval must be sought from the original reviewing REC and Research and Development (R&D) office(s).

9. Ethical Consideration

The study will be carried out in accordance with the World Medical Association Declaration of Helsinki (1964) and its revisions (Tokyo [1975], Venice [1983], Hong Kong [1989], South Africa [1996] and Edinburgh[2000]).

Favourable ethical opinion will be sought from an appropriate REC before patients are entered into this study. Patients will only be allowed to enter the study once either they have had the opportunity to review the patient information sheet and have provided written informed consent.

The CI, Dr Campbell Roxburgh will be responsible for updating the Ethics committee of any new information related to the study.

10. Finance and Indemnity

Funding for the study has been secured from the Beatson Cancer Charity. The study is sponsored by NHS Greater Glasgow & Clyde. The sponsor will be liable for negligent harm caused by the design of the trial. NHS indemnity is provided under the Clinical Negligence and Other Risks Indemnity Scheme (CNORIS).

11. Publications

The results may be disseminated through peer reviewed scientific journals and conference presentations. Patients will not individually be notified of the results given the parameters due to be evaluated are not known to be of clinical value at present. Should patients wish copies of any publications or presentations this can be provided on request as well as a lay summary of findings.

12. References

1. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL et al Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015 Jul 2;373(1):23-34.

2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA et al Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010 Aug 19;363(8):711-23.

3. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015 Apr 3;348(6230):124-8.

4. Masucci GV, Cesano A, Hawtin R, Janetzki S, Zhang J et al. Validation of biomarkers to predict response to immunotherapy in cancer: Volume I - pre-analytical and analytical validation. J Immunother Cancer. 2016 Nov 15;4:76.

5. Meng X, Huang Z, Teng F, Xing L, Yu J. Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy. Cancer Treat Rev. 2015 Dec;41(10):868-76.

6. Taube JM, Klein A, Brahmer JR, Xu H, Pan X et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res. 2014 Oct 1;20(19):5064-74.

7. Michael-Robinson JM, Biemer-Hüttmann A, Purdie DM, Walsh MD, Simms LA et al. Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status. Gut. 2001 Mar;48(3):360-6.

8. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015;372(26):2509-20.

9. Overman et al. Nivolumab ± ipilimumab in treatment of patients with metastatic colorectal cancer with and without high microsatellite instability: CheckMate-142 interim results. JCO 2017 (abstract ASCO GI)

10. Shahabi V, Postow MA, Tuck D, Wolchok JD. Immune-priming of the Tumor Microenvironment by Radiotherapy: Rationale for Combination with Immunotherapy to Improve Anticancer Efficacy. Am J Clin Oncol. 2015 Feb;38(1):90-7.

11. Lim SH, Chua W, Cheng C, Descallar J, Ng W et al. Effect of neoadjuvant chemoradiation on tumor-infiltrating/associated lymphocytes in locally advanced rectal cancers. Anticancer Res. 2014 Nov;34(11):6505-13.

12. Shinto E, Hase K, Hashiguchi Y, Sekizawa A, Ueno H et al. CD8+ and FOXP3+ tumor-infiltrating T cells before and after chemoradiotherapy for rectal cancer. Ann Surg Oncol. 2014 Jun;21 Suppl 3:S414-21

13. Roxburgh C, Vakiani E, Shia J, Daniels T, Weiser MR. Investigation into the Immune priming effect of FOLFOX chemotherapy in locally advanced rectal cancet. Cancer Immunol Research 2016 (abstract)

14. Sutton P, Evans J, Jones R, Malik H, Vimalchandran D et al. Proteomic analysis to identify biomarkers in the primary tumour that predict response to neoadjuvant chemotherapy in liver metastases. Lancet 2015;385(suppl 1):S95

Appendix IV : Patient Satisfaction Questionnaire

FLEXIBLE SIGMOIDOSCOPY PATIENT SATISFACTION QUESTIONNAIRE

TIMEPOINT:	Strongly Agree	Agree	Not Sure	Disagree	Strongly Disagree
Convenience and Accessibility					
I felt that I had to wait too long					
The service is a place that is easy for me to get to					
I found it hard to find a convenient time to come to the service					
Staff Interpersonal Skills					
I felt free to ask the staff questions I wanted to ask					
The staff seemed to hurry me through too quickly					
The staff used words that were hard to understand					
Physical Surroundings					
I had enough privacy while the sigmoidoscopy was being done					
Perceived Technical Competence					
The physician was too rough when performing the sigmoidoscopy					
I feel confident the sigmoidoscopy was performed properly					
Pain and Discomfort					
I had a lot of pain during the sigmoidoscopy					
The flexible sigmoidoscopy was more comfortable than I expected					
The sigmoidoscopy caused me great discomfort					
The sigmoidoscopy was worse than my first sigmoidoscopy					
Expectations and Beliefs					
I was very anxious about having the flexible sigmoidoscopy					
I was embarrassed by the sigmoidoscopy					
Undergoing sigmoidoscopy will benefit my health					
General Satisfaction					
I was very satisfied with the treatment I received					
I would strongly recommend a sigmoidoscopy to my friends					
I would be willing to have another if necessary					
I was happy to undergo the additional sigmoidoscopy for research purposes					

Appendix III

Chief investigator: Dr Campbell Roxburgh

Tel: 01412018676 or 01412018527

E-mail: campbell.roxburgh@glasgow.ac.uk

PATIENT CONSENT FORM Version 2 18/01/2018

Title of Project: An investigation into the potential immune priming effect of chemotherapy and radiotherapy on the tumour microenvironment in rectal cancer

Lay title: Investigating the immune response to radiotherapy in rectal cancer

Please initial box

I confirm that I have read and understand the information sheet dated....18/01/18 (version 2) for the above study. ☐

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

I understand that sections of my medical notes and my study information may be looked at by the research team and representatives of the study Sponsor (NHS GG&C) where it is ☐

relevant to my taking part in the research. I give my permission for this access to my information.

I agree to allow blood samples to be taken for study purposes during my treatment and for the examination of these samples in the laboratory for the purposes of this study. ☐

I agree to allow extra tumour samples to be taken in endoscopy or clinic during my treatment and for these to be examined in the laboratory for the purposes of this study ☐

I agree that any surplus blood or tissue samples not used in the laboratory examinations as part of this research study can be stored for potential future research. All future work will be ethically approved. ☐

I agree to take part in the above study. ☐

_____ _____ _____

Name of subject/Participant Number **Date** **Signature**

Name of researcher Date Signature _____