Determination of prognostic factors in early rectal cancer and the implications for rectal preservation surgery

Helen J S Jones MBChB, FRCSEd

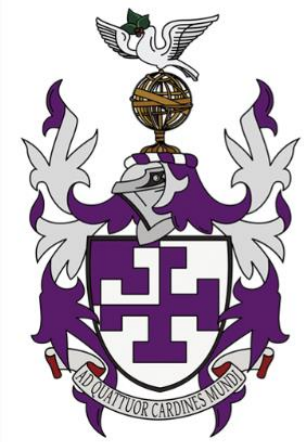
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Abstract

Local excision of early rectal cancer has benefits to patients, with reduced morbidity compared to radical surgery, but carries a higher risk of local recurrence. A detailed analysis of contemporary outcomes after local excision by TEM is presented. Currently, a coarse estimate of the recurrence risk is made on routine histopathological features and patients are advised regarding the options for subsequent management: radical surgery is the current standard if high risk; adjuvant radiotherapy is used in some places to reduce risk but is unproven; or close surveillance. Better, more accurate, and individualised means of predicting recurrence risk are sought to aid this decision process in this clearly defined group. Potential prognostic factors addressed in this work are sentinel lymph node biopsy (Chapter 3), more detailed histopathological analysis including routine mutation panel testing (Chapter 4), and digital pathology assessment and gene expression (Chapter 5). Areas of promise are perineural invasion, absence of common mutations, artificial intelligence-based assessment of the ratio of desmoplastic to inflamed stroma, and expression levels of certain genes, particularly those involved in the immune response. A search is also made for potential indicators of responsiveness to radiation which would help guide the decision to use this modality and spare patients with unresponsive tumours from potentially harmful exposure; this may also be of benefit to those with more advanced cancers considering neoadjuvant treatment, and some consideration is given to this group. Some of the early cancers display transition from adenoma, and differential molecular features that may cast light on this process are sought (Chapter 6). Finally, an assessment of functional outcomes after various rectal-preserving pathways is also made (Chapter7). More accurate and clinically practical recurrence prediction can be made now using readily accessible information, and further work on some of the gene signatures identified could improve this still further.

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Finally, I must record my gratitude to the patients who have contributed by consenting to various studies and completing questionnaires, and who are the ultimate inspiration for this work.

Declaration

I, Helen Jane Shearer Jones, declare that the work on which this thesis is based in my original work and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. The work is original except where indicated by reference in the text.

Signed: Date:

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List of abbreviations

ACPGBI	Association of Coloproctology of Great Britain and Ireland
AI	artificial intelligence
APE	abdomino-perineal excision
AUC	area under the curve
CAF	cancer-associated fibroblast
CEA	carcinoembryonic antigen
CI	confidence interval
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CRF	case report form
CRIS	colorectal cancer intrinsic subtype
CMS	Consensus molecular subtype
CRC	colorectal cancer
CRF	case report form
CRT	chemo-radiation
ctDNA	circulating tumour DNA
DFS	disease-free survival
DNN	deep neural network
ECM	extracellular matrix
EMR	endoscopic mucosal resection
EMT	epithelial-mesenchyme transition
EMVI	extramural vascular invasion
ERUS	endorectal ultrasound
ESD	endoscopic submucosal dissection
EV	extracellular vesicle
FC	fold change
FDR	false discovery rate
FFPE	formalin-fixed paraffin-embedded
GCP	Good clinical practice

GO	gene ontology
GSEA	gene set enrichment analysis
H&E	Haemoatoxylin and eosin
HR	hazard ratio
ICG	indocyanine green
ICGI	Institute for Cancer Genetics and Informatics, Oslo
ICH	International Council for Harmonisation
IIEF-5	international index of erectile function (5 item)
IPSS	international prostate symptom score
IQR	inter-quartile range
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LAR	low anterior resection
LARS	low anterior resection syndrome
LD	ligand dependent
LE	local excision
LI	ligand independent
LR	local recurrence
LVI	lymphovascular invasion
MDT	multi-disciplinary team
MFSQ-9	McCoy female sexuality questionnaire (9 item)
MMR	mismatch repair
MRI	magnetic resonance imaging
MSI	microsatellite instability
NAR	neoadjuvant rectal score
NIR	near infrared
NPV	negative predictive value
NTP	nearest template predictor
OCHRe	Oxford Centre for Histopathology Research
OS	overall survival
OUH	Oxford University Hospitals NHS Foundation Trust
PPI	protein-protein interaction

PPV	positive predictive value
QOL	quality of life
RCP	Royal College of Pathologists
REC	Research ethics committee
ROC	receiver operating characteristic
SD	standard deviation
SLN	sentinel lymph node
SLNB	sentinel lymph node biopsy
TAMIS	transanal minimally invasive surgery
TaTME	transanal total mesorectal excision
TEM	transanal endoscopic microsurgery
TMA	Trust management approval
TME	total mesorectal excision
TNT	total neoadjuvant treatment
TSA	traditional serrated adenoma
TSR	tumour stromal ratio
TVA	tubulo-villous adenoma
VAS	visual analogue scale

Chapter 1: Introduction and Background

1.1 Rectal cancer

Rectal cancer is a common problem, with some 11,000 new patients diagnosed each year in the UK[1]. With more public awareness and screening programmes rectal cancer is often now detected at an earlier stage[2]. The traditional treatment involves radical surgery, with life-altering impacts for the patient. Ideally, early, or less aggressive, cancer would be managed by less invasive treatment while achieving good oncological outcome but currently most patients with early-stage disease still undergo radical surgery.

Local excision, with rectal preservation, is recognised as an effective treatment for early rectal cancer, but as it does not remove the mesorectum which may harbour undetected metastases, there is an increased risk of recurrence. It is important to clarify this risk as much as possible, so patients can be given accurate information in order to make informed decisions about further management.

1.1.2 Rectal cancer is not the same as colon cancer

Rectal cancers make up about a third of colorectal cancer but have some unique properties[3,4]. Most studies looking at pathogenesis or risk stratification consider colorectal cancer in general, and such findings may not be relevant to rectal cancer.

The location of the rectum means tumours are more likely to cause symptoms such as bleeding at an earlier stage. Additionally, the rectum is easily accessible to endoscopic examination to assess symptoms or for screening. Being deep in the pelvis, in close contact with vital structures, makes surgical access more challenging. It does, however, make the rectum amenable to radiotherapy. Low tumours may require removal of the anal canal, leaving the patient with a permanent stoma which some may be very keen to avoid.

Most of the venous drainage follows the inferior mesenteric vein to the portal vein and liver, however, the extensive venous plexus around the rectum also allows communication with the systemic system via the internal pudendal vein. In consequence, while the liver is the most common site for metastases from colorectal tumours, rectal cancers have a higher incidence of metastases to the lungs. On the other hand, metastases within the abdomen are less common from rectal cancers, as most of the rectum has no peritoneal covering[4].

At the molecular level, rectal cancers also show differences[3]. They generally have low rates of mutation and tend to display chromosomal instability, with aneuploidy and loss of heterozygosity, or specific methylation (*MGMT* rather than *MLH1*). Such differences have implications for the assessment of prognostic features and highlight the need for studies focussed on rectal cancer.

1.2 Treatment challenges

The traditional surgical approach for rectal cancer involves a radical resection of the rectum and surrounding mesorectum, which contains the lymph nodes. Total mesorectal excision (TME) is known to offer the best long-term local and distant disease control; this may be low anterior resection (LAR) or abdomino-perineal excision (APE) depending on tumour factors, such as location, and patient factors including preference and ability to cope with a stoma.

These procedures carry a risk of perioperative morbidity and mortality as well as long-term sequelae of impaired bowel function or permanent colostomy, and impaired urinary and sexual function. These are important considerations especially as the median age at diagnosis is 69 and around 30% of patients are aged over 80[1]. Organ-preserving local resection is better tolerated by patients with low perioperative morbidity but is associated with higher rates of local recurrence: 12.5% vs 6.9% at 5 years for T1 tumours and 22.1% vs 15.1% for T2[5].

Local excision will be curative if the cancer is completely removed and has not metastasised. Problems arise because the cancer may be more advanced or aggressive than is anticipated based on information available prior to surgery.

1.2.1 Staging

Prior to treatment, assessment is made by clinical examination, endoscopic visualisation, imaging with magnetic resonance imaging (MRI) and endorectal ultrasound (ERUS), and histopathological assessment of a biopsy. Histopathology of a biopsy may be limited to confirmation of malignancy or may provide further information such as degree of differentiation and presence of lymphovascular invasion. There is scope for obtaining more prognostic information at this stage if a reliable feature could be identified.

1.2.1.1 Imaging

ERUS can assess the depth of tumour invasion, theoretically allowing differentiation of T1 tumours that are confined to the submucosa from T2 tumours that penetrate the muscularis propria. A Norwegian study[6] found ERUS had 88% accuracy in distinguishing adenoma from early cancer, better than MRI (75%), however it tended to overstage T1 tumours as T2-3.

High-resolution MRI scanning is the best imaging modality currently available, but it is not completely reliable. Important features that the MRI addresses are depth of tumour invasion (T stage), involvement of lymph nodes (N stage), extramural venous invasion (EMVI), and whether the circumferential resection margin (CRM) is threatened by tumour. Accurate determination of lymph node involvement is particularly difficult: size alone cannot be used as a criterion in rectal cancer as even very small nodes may contain malignancy. Wang *et al.*[7] found 52% of mesorectal lymph nodes between 5-10 mm diameter in TME specimens with rectal cancer were infiltrated with

tumour. In April 2014, the Dutch guidelines for classifying lymph nodes as positive on MRI became more restrictive. Previously, nodes over 5mm in diameter were defined as suspicious, whereas now nodes are defined as suspicious if over 9mm, or 5-9mm and possessing two of three possible malignant features, or under 5mm and possessing all three of: round form, irregular margin and heterogenous texture[8]. This resulted in fewer patients receiving neoadjuvant treatment but had no effect on outcomes at 1 year.

Unpublished Oxford data based on 57 patients with T1/2 rectal cancer found MRI was 84% accurate for nodal status compared with histopathology, in keeping with published results: Balyasnikova *et al.*[9] found MRI 84% accurate compared with histopathology for lymph node involvement in the TME specimen, and 89% accurate in distinguishing T1 sm1/2 stage from more invasive cancers.

EMVI is defined as tumour cells within the veins outside the muscularis propria and is associated with poor prognosis[10]. Zhang *et al.*[11] demonstrated that EMVI can be reliably identified on MRI by experienced radiologists and is associated with local and distant recurrence, and poorer survival. Future technical advances will further increase the accuracy of pre-treatment imaging.

Another potential staging tool is sentinel lymph node biopsy, using contrast material to identify then biopsy the lymph node draining the cancer. This is effective in other cancers, such as breast, but has not yet been successful in rectal cancer.

1.2.2 Local excision

In the past, local excision (LE) meant open transanal excision, but the limited access often results in a poor-quality specimen and poor patient outcomes. In 1983, Buess *et al.*[12] introduced transanal endoscopic microsurgery (TEM); this is the technique most commonly used in Oxford for LE. The specialised instrumentation provides optimal visualisation, a stable pneumorectum, and access as far as the rectosigmoid junction. Even so, uptake among colorectal surgeons was initially slow.

More recent technical developments such as TAMIS improved manoeuvrability within the rectum and have led to a rapid uptake in LE for rectal cancer among colorectal surgeons. These modern techniques result in better outcomes, as there is less tumour fragmentation and an improved rate of clear margins[13].

LE has lower perioperative morbidity and mortality, and a quicker recovery with shorter hospital stay and reduced healthcare costs. It preserves the rectum and anal sphincters so retains relatively normal bowel function, whereas radical surgery often involves a permanent stoma or can be followed by 'low anterior resection syndrome' which many patients find distressing. Preservation of pelvic nerves avoids the risk of impaired urinary and sexual function. These benefits, however, need to be balanced against the possible oncologic compromise due to leaving tumour cells in the mesorectum and lymph nodes. While patients with an early tumour may be attracted to the option of LE, it is essential that they are counselled and understand the higher risk of local recurrence, 16% in the UK TEM database[14], and what that may entail. Better prognostic information would remove some of the guesswork in weighing these options. When balancing quality of life with long-term survival, the patient's and surgeon's opinions may differ. Different surgeons and patients are willing to accept different recurrence rates and view the risks in different ways. A 20% recurrence risk with LE could also be viewed as an 80% chance of no recurrence without exposure to the potential morbidity of major surgery.

Another consideration is whether LE will compromise later radical surgery should it become necessary, either because of unexpected adverse histopathological features in the tumour, or as a salvage procedure for local recurrence.

1.2.2.1 Local recurrence

When local recurrence occurs salvage surgery may be possible. Successful salvage requires early detection of recurrence, so a surveillance regime is critical. Bikhchandani *et al.*[15] studied 27 patients

who had salvage surgery. The median time till recurrence was just over a year, and in most patients, recurrence was luminal while six had nodal recurrence only. Salvage surgery may require pre-operative chemoradiation and a more extensive operation than would have been required as a primary treatment, due to disruption of tissues planes and scarring or to the extent of the recurrent disease. In Bikhchandani's cohort the overall 5-year survival after salvage was 50%. Six of the cohort subsequently developed distant metastases. These figures indicate that developing recurrent cancer after LE disadvantages patients who may otherwise have been cured by primary radical surgery. The high incidence of metastatic disease with these initially early stage tumours, considered suitable for LE, highlights that those suffering recurrence may have inherently more aggressive cancers that are not identified by current prognostic markers. Alternatively, treatment by LE, if resulting in incomplete resection, may leave the disease in situ for longer, giving a longer period for metastases to develop.

1.2.3 Management pathways after local excision

Following LE, histopathological assessment of the tumour will detect higher-risk features associated with an increased risk of positive lymph nodes[16]. If these features are found, ACPGBI guidelines[17] recommend completion radical surgery to reduce the risk of local recurrence. This subjects the patient to all the risks of radical surgery that they previously hoped to avoid. Other options to mitigate the risk of local recurrence are close surveillance and radiotherapy followed by surveillance. There is not yet strong evidence to support the use of adjuvant radiotherapy in this setting, but it has been increasingly used locally with promising results. One issue is that some tumours are not sensitive to radiation but at present these cannot be identified, so some patients receive radiation with no benefit.

These three options all carry their own risks and benefits and it is essential the patient clearly understands these before coming to a decision.

1.2.3.1 Surveillance

Close surveillance involves a protocol, developed locally, of endoscopy and MRI scans 3-4 monthly for 2-3 years then 6-monthly till 5 years, and annually thereafter if deemed appropriate. This strategy relies on detecting any recurrence early when it is amenable to the simplest salvage treatment. It is the least interventional strategy but involves the most uncertainty for patients and some can find the repeated attendance stressful. However, as most locally excised cancers do not recur the strategy avoids subjecting many patients to unnecessary additional therapy.

To be effective, surveillance requires a patient who understands the need for ongoing follow-up and is willing to comply with the regime, and a system with effective and timely recall that can accommodate the necessary appointments. This regime is not suited to all patients; some find it too stressful.

1.2.3.2 Outcomes

Verseveld *et al.*[18] analysed the Netherlands Cancer Registry data from 2005-2015 and compared primary radical surgery with completion surgery and LE alone, excluding patients who had (neo)adjuvant treatment. Relative survival at 5 years (ratio of the observed survival rate to expected rates for general population) for T1 was 95% for primary TME, 93% for LE alone and 85% for completion. For T2 survival was around 90% for all groups while for T3/4 survival after primary or completion TME was around 74% but only 20% for LE alone. Leijtens *et al.*[19] compared outcomes for T2/3 cancers excised by TEM followed by surveillance alone or completion surgery and found a local recurrence rate of 35% in the surveillance group compared with 11% for completion surgery; distant metastases occurred in 16% of both groups. They found similar disease-specific survival at 3 years in both groups (91 – 93%) but the overall survival was 63% with surveillance and 91% after

completion surgery. The surveillance group had a median age of 81 compared to 65, and more comorbidity. They suggest considering surveillance alone in the frail and elderly.

While LE alone was previously only considered 'safe' in those with early, low-risk T1 cancers, the more recent evidence challenges this. Several trials, including STAR-TREC[20] and TESAR[21], are now exploring how structured surveillance and pre- or post-operative radiotherapy can extend the range of tumour size and stage that can safely be removed by LE, permitting more patients to benefit from rectal preservation.

1.3 Stratification of rectal cancer

Several stratification systems have been developed for colorectal cancers. Other than routine histopathology, a few mutations and MSI status, these are mainly used as a research tool rather than contributing to patient management. The aim of stratification is to identify tumours, which display similar characteristics and may be expected to behave similarly in response to various treatment strategies. Accurate and detailed stratification achieved prior to commencing treatment would allow a specific regime to be targeted to those tumours which were most likely to respond and avoid unnecessary or harmful treatment in those who will not significantly benefit. At present, pre-treatment stratification is primarily dependent on staging with MRI. Although there has been much intensive research into biomarkers over recent years, no effective means has yet been found. Dayde *et al.*[22] postulate this is due to researchers looking at only a single modality rather than combining molecular, stromal and immune features with clinical, imaging and histopathological features. And as colorectal cancer is a heterogenous disease, with several different carcinogenesis pathways, there is unlikely to be a single prognostic feature common to all tumours.

1.3.1 Histopathology

Following excision of the tumour, routine histopathology provides the next level of stratification. The Royal College of Pathologists (RCP) has set out the core data items that should be reported for rectal cancers[23]. The reporting requirements for locally excised tumours (Table 1.1) are more detailed than for radical resections to inform decisions about any further treatment.

TABLE 1.1 RCP CORE DATA ITEMS FOR LOCALLY EXCISED RECTAL CANCERS

RCP Core data items for locally excised rectal cancers
Specimen type (polypectomy, EMR, ESD or TEM)
Site of tumour
Overall specimen size
Histological tumour type (mucinous or signet ring are higher risk)
Histological differentiation
Extent of local invasion
Venous invasion
Lymphatic invasion
Perineural invasion
Presence of a precursor adenoma (or rarely other polyp type)
Margin involvement by carcinoma (deep/peripheral)
Minimum deep margin clearance of the invasive carcinoma (in millimetres)
pT stage
MSI/MMR tumour status

DEPTH OF INVASION: T1 indicates the tumour is confined to the submucosa, while T2 invades the muscularis propria. T3 reaches the outer layer of the bowel wall. For T1 cancers, further subdivision is made. The Kikuchi levels sm1-3 are used for sessile tumours; this system divides the submucosa into thirds. The incidence of lymph node metastases increases with depth: 2% for sm1, 8% for sm2 and 23% for sm3[24]. For polypoid lesions, the Haggitt system reports the depth of invasion into the stalk. Another option measures the depth of invasion beyond the muscularis mucosae, with a cut-off of 1mm marking higher-risk disease[25].

LYMPHATIC AND VENOUS INVASION: Lymphovascular invasion (LVI) refers to cancer cells in lymphatic or vascular channels. Lim *et al.*[26] identified LVI in 25% of over 2,400 colorectal cancers.

LVI correlated with older age, poor differentiation, more advanced tumours, more lymph node metastases and lower 5-year disease-free survival: 57% vs 83%. The RCP recommends reporting lymphatic and venous invasion separately as metanalyses have shown lymphatic invasion to be a more powerful predictor of lymph node metastases than venous invasion[27]. The extent of vascular invasion is also important: Betge *et al.*[28] identified EMVI as the key prognostic feature in colorectal cancer. A metanalysis of EMVI in rectal cancer[29] found the incidence of detected EMVI ranged from 9-61%, with an overall prevalence of 26% and a 5-year overall survival of 40% for EMVI positive patients. They also note that MRI could be more accurate that histopathology in detecting EMVI, and it assesses the whole rectum rather than just the excised specimen.

PERINEURAL INVASION: Ueno *et al.*[30] reviewed almost 1,000 colorectal cancers and found the 5-year disease-free survival significantly correlated with the extent of perineural invasion: 88% with no invasion, 70% for intramural invasion and 48% for extramural invasion. They also noted that the inter-pathologist agreement for the grading of perineural invasion was more accurate than for venous invasion.

RESECTION MARGIN INVOLVEMENT: local excision may involve fragmentation of the specimen. This is more likely in polypectomy or transanal excision than TEM and may involve lateral and/or deep margins. The margin is usually only considered clear if tumour cells are at least 1mm away. However more recently it has been suggested that a margin of <1mm may be satisfactory in the absence of other poor prognostic features. Brown *et al.*[31] noted no residual carcinoma in 13 cases where the margin was negative with <1mm clearance.

MSI (MICROSATELLITE INSTABILITY): MSI occurs when any of the four DNA mismatch repair (MMR) genes are defective*: MLH1*, *MSH2*, *MSH6* and *PMS2*. In Lynch syndrome this is due to a gene mutation while in sporadic cancers it is due to hypermethylation affecting *MLH1*[32]. These tumours have a better prognosis with lower tendency to metastasise[23]. MSI in only found in about 2% of

rectal cancers[33] and Hong *et al.*[34] found that while prognostic in colon cancers, it was not useful in rectal cancers.

TUMOUR BUDDING: This refers to clusters of 1-4 tumour cells in tumour stroma. It is associated with aggressive tumours and poor prognosis. It is not currently an RCP core data item because of lack of consensus about its assessment and relatively low inter-pathologist reporting reproducibility[35]. However, an international conference has now made recommendations[36]. Budding is associated with lymph node metastases[37]. Koelzer *et al.*[35] looked at molecular characteristics of tumour budding and found features that promote epithelial-mesenchymal transition (EMT) such as activation of the Wnt signalling pathway as well as evidence of weak immune infiltration around the tumour, suggesting that cytotoxic T cells destroy tumour budding cells.

WIDTH OF INVASIVE FRONT: A study by Toh *et al.*[38] suggests that the width of the invasive front of the tumour, with a cut-off of 11.5mm, and the area of submucosal invasion, cut-off: 35mm^2, is a better predictor of lymph node metastases than depth of invasion. They hypothesise that a greater invasive area increases the chances of tumour encountering a lymphatic vessel.

PERITUMORAL INFLAMMATION: This also is a non-core data item. Increased immune infiltrate is generally associated with better prognosis. Funada *et al.*[39] studied the peritumoral infiltration with CD8+ (cytotoxic) T cells and macrophages at the invasive front of colorectal cancers. They found low macrophage infiltration correlated with greater depth of invasion, more vascular invasion and more lymph node metastases. Low cytotoxic T cell infiltration correlated with shorter overall survival.

1.3.2 Molecular biomarkers

Much current research is focussing on molecular features that could provide prognostic information about the tumour. A wide variety are being explored that cover the three distinct compartments of the tumour: 1) epithelial tumour cells, including genetic and epigenetic features; 2) stroma and fibroblasts; and 3) immune infiltrate. The remainder of this chapter reviews these features, with a focus on areas investigated in this thesis which is targeted to markers potentially applicable in early rectal cancers where the aim is organ preservation by local excision with or without radiotherapy. Ideally such markers would be simple, reliable, not costly and suited to small biopsy or local excision specimens. This section provides the background for Chapters 5 and 6.

COLORECTAL CARCINOGENESIS PATHWAYS

There are three molecular carcinogenesis pathways in colorectal cancer that reflect different genetic and epigenetic features: 1) chromosomal instability (CIN); 2) MSI, mentioned above; and 3) CpG island methylator phenotype (CIMP) or epigenetic instability pathways (not considered here). Bae *et al.*[40] also describes the two morphological pathways: 1) adenoma-carcinoma sequence; and 2) serrated neoplasia pathway. Compared with the colon, the rectum has low levels of CIMP and MSI tumours, and low levels of *BRAF* and *KRAS* mutations[41]. Current molecular stratification in colorectal cancers is mostly confined to MSI, and *KRAS* and *BRAF* mutations.

1.3.2.1 Tumour cell features

Tumour cell markers may be based on genetic features such as differential expression of genes, mutations and copy number variation. However, much of the abnormal behaviour of a cancer cell is determined by epigenetic features. These are non-genetic features, such as methylation of DNA and organisation of chromatin, that influence gene expression and may promote carcinogenesis for example by inactivating tumour suppressor genes, activating oncogenes or promoting chromosomal instability. The genetic changes that lead to the development of colorectal cancer

are varied and heterogenous. A common factor is inactivation of several tumour suppressor genes, such as *APC* and *TP53*.

GENE SIGNATURES

Many groups have sought a gene signature of differentially expressed genes that could be used to stratify tumours according to prognosis or sensitivity to radiation. Many signatures have been published over recent years but with little overlap. Park *et al.*[42] compared five genetic prediction signatures and found that only a few genes occurred on more than one gene list. The concordance between the five signatures for assigning patients to high and low risk categories varied from 4% to 36%, and only two of the five robustly identified poor prognosis patients in independent cohorts.

CHROMOSOMAL INSTABILITY

PLOIDY: Ploidy refers to the number of sets of chromosomes in a cell nucleus. Most human cells are normally diploid. Chromosomal instability (CIN) can result in tumour cells with an abnormal DNA content, known as aneuploidy, whereby some chromosomes are replicated or deleted, either in part or in whole. In general, aneuploidy in cancer cells is associated with a worse prognosis. Goh *et al.*[43] found 64% of 203 rectal cancers were aneuploid and showed an independent but small predictive effect of aneuploidy on survival. Flow-based cytometry has now been replaced by automated image-based cytometry, allowing more accurate and detailed assessment of ploidy. The DNA ploidy status of tumour cells in early ovarian cancer predicts which patients will benefit from adjuvant chemotherapy after surgical removal and is used routinely in some centres to aid decision-making[44]. Danielsen *et al.*[45] reviewed the role of ploidy assessment. They note that as most advanced-stage cancers are aneuploid, useful results can only be determined in studies that provide tumour stratification by stage. They tabulated 17 published studies of colorectal cancer where there was sufficient data to assess outcomes in stage II cancer. Most found a significant association between aneuploidy and survival, but aneuploidy was only independently predictive in studies with over around 100 patients. Their review highlighted three large-scale studies that used

image-based cytometry: Sinicrope *et al.*[46] looked at the prognostic benefit of ploidy and MSI and found the 5-year survival for MSI and for diploid non-MSI tumours was significantly better than for aneuploid tumour. Mouradov *et al.*[47] studied over 1,000 colorectal cancers, looking at aneuploidy, MSI and mutations in several genes including *KRAS* and *BRAF*. They found both aneuploidy and MSI were independent predictors of outcome at 5 years, but the individual mutations were not. They also noted that a semi-quantitative measure of CIN was a better predictor than a binary measure. The third study, Hveem *et al.*[48] again looked at CIN and MSI in almost 1,000 colorectal cancers and found that both were independent predictors of recurrence in stage II cancers. Danielsen *et al.*[45] conclude that ploidy measured by automated image-based cytometry is relatively simple, inexpensive and reproducible, so could be widely used to increase the prognostic information available after cancer resection.

CHROMATIN STRUCTURE: The organisation of the chromatin in the cell nucleus has been found significant in cancer development. Chromatin structure affects gene expression[49]. Schuster-Böckler and Lehner[50] found that the arrangement of the genome into domains where the chromatin structure was heterogenous or not had a dominant influence on variation in regional mutation frequency. In the heterogenous areas the chromatin is densely packed with strong short-range interactions between molecules making the chromatin inaccessible. In contrast, in euchromatin areas, the chromatin has a more open arrangement and is accessible. Kleppe *et al.*[51] reported the use of an automated technique using machine learning algorithms to analyse stained nuclei for chromatin organisation. They developed and validated a marker for chromatin heterogeneity, named nucleotyping, in over 1,000 colorectal cancers and a range of other cancer types. Using this marker to assign cancers to a binary classification as chromatin heterogenous or homogenous they found that heterogeneity was an independent predictor of worse cancer-specific survival, with a hazard ratio of 1·9 (95% CI: 1·3–2·8) compared with homogenous stage II colorectal cancers.

1.3.2.2 Stroma and fibroblasts

The second tumour compartment is the stroma which contains, among other things, fibroblasts and extracellular matrix proteins that interact with the epithelial cancer cells, and so influence disease progression and metastatic potential of the cancer.

DESMOPLASTIC STROMA

Desmoplasia refers to disorganised production of connective tissue, comprising mainly collagen fibres and myofibroblasts. Coulson-Thomas *et al.*[52] found that collagen types I, IV and V were upregulated in the desmoplastic stroma of colorectal cancers. They also noted that direct contact between cancer cells and stromal fibroblasts led to an increase in the density of the extra-cellular matrix (ECM) which involved disorganised collagens and proteoglycans (fibromodulin, versican, perlecan and biglycan). Ueno *et al.*[53] looked at the desmoplastic reaction of the stroma at the advancing edge of the tumour on H&E slides from 880 colorectal tumours. They categorised the reaction into mature, intermediate and immature, with the 'immature' category showing disorganised thick keloid-like bundles of hyalinised collagen and myxoid stroma. They found immature stroma to be an independent predictor of poor prognosis. They also noted less immune response in immature stroma, with fewer cytotoxic (CD4+) T cells and tumour-Infiltrating macrophages (CD68+).

TUMOUR-STROMAL RATIO

The tumour-stroma ratio (TSR) is the ratio of tumour (epithelial) tissue to stroma (connective tissue) in the cancer. In many types of epithelial cancer, a higher proportion of stroma in the cancer corelates with poorer prognosis. Van Pelt *et al.*[54] reviewed the role of the stroma in colorectal cancer. Cancer-associated fibroblasts (CAF) are the activated form of fibroblasts and are the main cell type in the stroma. These cells provide growth factors for the cancer cells and promote angiogenesis, supplying the tumour with blood-borne nutrients to promote cancer cell survival. They also influence immune cells in the local area to promote cancer cell survival. Hutchins *et al.*[55]

found high-stroma tumours, with ≥ 65% stroma, are more common in the rectum, 11%, than the colon, 5%.

Several studies have investigated the value of TSR as a predictor of outcome in colorectal cancer. Most of these have focussed on colonic cancer, for example Huijbers *et al.*[56] assessed 710 patients from the VIKTOR trial and found survival significantly worse in those with over 50% stroma. West *et al.*[57] included rectal cancers in their series, excluding those who had neoadjuvant treatment and did not find TSR to be significant in rectal cancer (30% of 145 tumours), in contrast to colonic cancer. Scheer *et al.*[58] looked at the value of TSR in rectal cancer in a retrospective study on 154 patients who had not had neoadjuvant treatment. They used three categories, based on microscopy of an H&E stain, defining low TSR as less than 30%, high as above 70% and intermediate between these. They found that the high and intermediate stroma groups had a significantly worse disease-free and overall survival than the low stroma group. They noted low interobserver agreement regarding the TSR grouping and suggested accuracy could be improved by using specific stains and digital pathology measurement. Some papers[54,58] raised the possibility of using TSR in pre-treatment biopsies to predict sensitivity to neoadjuvant treatment, but this has not yet been investigated.

Measurement of TSR is traditionally done using an H&E stained slide and visual measurement; newer automated digital techniques have been introduced but are not yet in widespread use. Hutchins *et al.*[55] used a digital method in over 1,800 colorectal cancers and found tumours with over 65% stroma had almost double the risk of recurrence (42% vs 22%) in 10 years compared with those with less than 50% stroma. TSR varies throughout the tumour so it is recommended to make the measurement at the deepest invasive front where the highest TSR is found[59,60]. Van Pelt *et al.*[54] note good interobserver agreement in the measurement in several studies. The Leiden University Medical Center has provided an e-learning tool for pathologists[61] to standardise TSR reporting, making a binary distinction between low and high stroma tumours with a cut-off at 50%. They have opened an international prospective study, UNITED, to validate the reproducibility of this TSR

method. If agreed by the TNM Evaluation Committee (UICC) and College of American Pathologists, TSR could be included in the TNM staging algorithm to improve prognostic information in colorectal cancer[54]. TSR is less useful following neoadjuvant treatment as radiation can promote fibrosis.

An assessment of the genetic features of high TSR tumours[62] showed that as well as various collagen genes, three genes were significantly upregulated: *THBS2* (thrombospondin 2), involved in the focal adhesion pathway; *COX7A1* (Cytochrome C Oxidase Subunit 7A1) in the myogenesis pathway; and *LGALS1* (galectin-1), in the EMT pathway and also an immune response inhibitor.

Eriksen *et al.*[63] looked at the combination of TSR and tumour budding and found a correlation between high stroma content and high levels of tumour budding in colonic tumours. TSR was a significant independent prognostic factor, but with no additional value to tumour budding.

Danielsen *et al.*[64] combined DNA ploidy and TSR assessment in 2,624 early colorectal cancers, of which 25% were rectal, with a cut-off of 50% to differentiate high- and low-stroma groups. Using image cytometry, they classified the tumours as diploid or non-diploid. While the ploidy marker alone was not a significant factor, in combination with TSR it allowed discrimination between the otherwise large low-stroma group (87% of the cancers) into groups of low and intermediate recurrence risk which has potential value in stratifying adjuvant treatment. They came up with three groups: 1) diploid and low stroma; 2) diploid and high stroma or non-diploid and low stroma; and 3) non-diploid and high stroma. This classification was a strong independent predictor of 5-year cancer-specific survival for stage II disease, with hazard ratios of 1.77 (95% CI: 1.13-2.77) for the intermediate group and 2.95 (95% CI: 1.73-5.03) for the high-risk group compared with the low-risk group. The classification was not, however, predictive in more advanced cancers.

STROMAL GENES

Calon *et al.*[65] found the stromal genes to be an important contributor to poor prognosis. They identified in three publicly-available datasets some 300 genes that were upregulated in high-risk, poor-prognosis subgroups of colorectal cancer (de Sousa E Melo's CCS3[66], Sadanandam's 'stem-

like' group[67] and Marisa's C4 subgroup[68]). They microdissected 13 colorectal cancer samples into epithelial and stromal cells and genetically profiled these separately. They found that the gene subset upregulated in the poor-prognosis tumours came from the stromal component, specifically the CAFs, rather than the epithelial cells. They note the importance of the TGF-β signalling pathway in the upregulated gene set. Isella *et al.*[69] came to the same conclusion on the source of the 'poor-prognosis' gene subset by developing a xenograft of human colorectal cancer cells in mouse stroma and found the poor-prognosis signal was no longer present.

1.3.2.3 Immune infiltrate

The third tumour compartment is the immune infiltrate. As already alluded to, poor immune response in the tumour microenvironment is associated with worse prognosis. Pagès *et al.*[70] developed the Immunoscore calculated from the average densities of memory CD45RO+ and cytotoxic CD8+ T lymphocytes, both in the tumour and at the invasive margin. They divided over 600 early colorectal cancers into four groups, low and high for each T cell types. They found significant differences in 5-year disease-free and overall survival with a recurrence rate at 5 years of 4.8% in the high/high group and of 75% in the low/low group. The score was an independent predictor on multivariate analysis. Mlecnik *et al.*[71] investigated a number of prognostic features in colorectal cancer and confirmed that the Immumoscore provided a powerful predictor of recurrence that was independent of MSI status. An international validation of a consensus Immunoscore in colon cancer[72] used the densities of CD3+ and CD8+ cells, measured by digital pathology. Recurrence at 5 years was significantly related to Immunoscore, occurring in 8% of those with high score, 14% with intermediate score and 23% with low score. Overall survival at 5 years ranged from 62% for low scorers to 82% for high scorers. They also noted that the Immunoscore was an independent predictor of recurrence and made the highest relative contribution to recurrence risk of the clinical parameters.

Hynes *et al.*[73] reviewed a variety of tumour microenvironment features in colorectal cancer. They evaluated H&E slides from 445 colon cancers to identify which features would be most feasible to incorporate into routine histopathology assessment to provide the most additional prognostic information. They found an association between cancer-specific death and three features: TSR, peritumoral lymphoid inflammation and Crohn's disease-like inflammation. They combined these into a 'fibroinflammatory score' and found that patients with at least two of: minimal peritumoral lymphoid inflammation, minimal Crohn's disease-like response and over 50% stroma in the tumour, were almost 2.5 times more likely to die from colorectal cancer than patients lacking these three features.

1.3.3 Colorectal cancer classification systems

Investigators have devised various classification systems to stratify colorectal cancer based on a number of the features mentioned above. A 2013 study[66] subdivided colon cancers into three Colon Cancer Subtypes (CCS). The first group showed CIN, the second MSI. The third group showed neither, but some showed CIMP and had a worse prognosis. They developed a four-protein staining panel (FRMD6, ZEB1, HTR2B and CDX2) to identify this third group. By looking at gene sets to differentiate precursor lesions they concluded that this poor-prognosis group arose from sessile serrated adenomas.

Two classification systems have now gained wide acceptance: the Consensus molecular subtype (CMS) and the Colorectal cancer intrinsic subtype (CRIS). Guinney *et al.*[74] reported on an international consortium that considered gene expression data from over 3,000 tumours in 18 datasets and devised a classification using 693 discriminant genes with four subtypes of colorectal cancer. The first, CMS1, termed 'MSI immune' makes up 14% of colorectal cancer and shows MSI, with high levels of CIMP and high frequency of mutations, often including *BRAF* mutation. This subgroup shows immune infiltration and activation and tends to have poor survival after relapse.

These tumours are more common in the right colon. The largest subgroup, CMS2, or 'canonical', makes up 37%. This subtype occurs mainly in the left colon and rectum and tends to have better survival rates after relapse. Molecular features include aneuploidy and strong activation of the Wnt and Myc signalling pathways. The third 'metabolic' subtype, CMS3, comprises 13% and shows metabolic dysregulation. Features include *KRAS* mutations, low CIMP and low levels of aneuploidy. CMS4 is termed 'mesenchymal' and comprises 23%. These show stromal infiltration, angiogenesis and strong TGF-β activation, and have the poorest outcome. In the rectum CMS1 is uncommon but the three other all occur frequently.

Becht *et al.*[75] looked at immune-specific gene expression in these subtypes. They found that CMS1 tumours over-expressed genes from cytotoxic T cells while CMS4 over-expressed genes related to CAFs, and angiogenic, inflammatory, and immunosuppressive signatures. They attribute the unexpected high immune signature in the poor-prognosis CMS4 tumours to inflammation induced by CAFs which impairs the usual beneficial effect of the abundant cytotoxic T cells.

Isella *et al.*[76] considered that many previous classification systems did not distinguish between the stromal and cancer cell components when investigating molecular features. They devised a model with cancer cells in mouse stroma so that they could examine genetic features deriving specifically from the cancer cells, allowing them to derive a classification intrinsic to the cancer. Their classification has five groups, known as CRIS (Colorectal Cancer Intrinsic Subtype) based on 565 genes expressed by the epithelial cells. Type A is mucinous, glycolytic and shows MSI or *KRAS* mutations. Type B shows strong TGF-β activation and EMT; these tend to be poorly differentiated and have poor prognosis. Both A and B tend to show MSI, CIMP, high frequency of mutations, mucinous histology, and occur mainly in the right colon. Type C shows increased EGFR signalling. Type D shows Wnt activation and increased *IGF2* expression. They found these tumours exhibited features of intestinal stem cells and have the best prognosis. Type E shows a Paneth cell-like phenotype and *TP53* mutation. Together C, D and E tended to show chromosomal instability and

aneuploidy. Both CMS and CRIS have been widely used in recent years to classify colorectal cancers. Alderdice *et al.*[77] specifically considered these classification systems in biopsy samples, to assess their potential as pre-treatment stratifiers, and found CRIS to be superior for giving the same result from different areas of the tumour and leaving fewer samples unclassified.

1.3.4 Serrated pathway cancers and TSA polyps

Most colorectal cancers arise through the adenoma-carcinoma sequence but up to 30% are thought to arise from the serrated pathway where the precursor polyp is a sessile serrated adenoma (SSA) or a traditional serrated adenoma (TSA)[78]. At endoscopy serrated lesions can be more difficult to diagnose and in the past were not always recognised as pre-malignant nor removed. A US study of over 14,000 polyps removed at endoscopy over 10 years found 4% were serrated lesions, of which 9% were TSA[79]. The incidence of serrated polyps increased after 2010 due to a change in practice to remove them. The molecular features that characterise these lesions include activating mutations in the RAS-RAF-MAPK pathway, *BRAF* or *KRAS* mutations and CIMP. Those that occur in the rectum tend to have *KRAS* mutations and not be MSI or CIMP. Mucin production and poor differentiation are common in serrated pathway cancers, and they tend to be associated with poor prognosis.

TSAs are characterised by the presence of ectopic crypt foci[80]. Sekine *et al.*[81] studied genetic alterations in various colorectal polyps, with a focus on TSAs. They found *RSPO* fusions (specifically *PTPRK-RSPO3*) in 31% and *RNF43* mutations in 24% of TSAs but not in non-serrated polyp types. *RSPO* and *RNF43* are both components of the Wnt pathway. They then examined more TSAs and found *RSPO* fusions in 33%, most commonly *PTPRK-RSPO3* but also several less common types[82]. These fusions resulted in overexpression of *RSPO3* and, to a lesser extent, *RSPO2*. In a further 3% of TSAs they observed overexpression of *RSPO* without underlying *RSPO* fusions. They also noted that overexpression of *RSPO* was mutually exclusive of other Wnt pathway gene mutations. The

RSPO fusion TSAs showed higher-grade dysplasia, suggesting these polyps are more likely to progress to cancer. Sheshagiri *et al.*[83] also showed that fusions involving *RSPO2* and *RSPO3* are mutually exclusive of *APC* mutations, leading them to conclude that these fusions provide an alternative pathway for Wnt activation and carcinogenesis. Most mutations that activate the Wnt pathway, for example in *APC* and *CTNNB1*, are ligand-independent (LI), meaning that the mutation causes activation of the pathway downstream, leading to cell proliferation, regardless of whether Wnt proteins bind to the cell. However, the pathway activated by *RSPO* fusions and *RNF43* mutations is ligand-dependent (LD), meaning the Wnt pathway is overstimulated at the cell surface[84].

1.4 Aim of thesis

The aim of this thesis is to investigate potential prognostic indicators in early rectal cancer that could be applied in clinical practice to provide an indication of the cancer's aggressiveness and responsiveness to radiation to allow more informed decision making about the suitability of an rectal-preserving approach in a particular patient, and if local excision is pursued, the most appropriate subsequent management pathway. Detailed aims are given in each chapter.

1.4.1 Thesis plan

Chapter 2 describes the methodology for most of the work covered in this thesis; some specific points are addressed in the relevant chapters.

Chapter 3 presents the results of a clinical trial assessing a potential novel method of sentinel lymph node identification in rectal cancer.

In chapter 4 the Oxford TEM database, containing over 200 patients who have had TEM for rectal cancer over an 11-year period, is analysed to assess current outcomes, particularly for the three

alternate post-TEM management pathways, and potential clinical and histopathological prognostic factors. These results provide a baseline for future work and enable comparison with published results.

Chapter 5 presents a laboratory-based analysis of the rectal cancer specimens from most of the patients covered in Chapter 4. A variety of digital and molecular techniques are used, with the aim of identifying factors prognostic for recurrence or responsiveness to radiation.

Chapter 6 continues the laboratory-based analysis, focussing on the molecular aspects of the transition from adenoma to carcinoma in a subset of the rectal cancers where the specimen contains both polyp and early cancer.

The final experimental chapter, 7, looks at a study that involved establishing a rectal cancer biobank for newly diagnosed tumours with the aim of identifying factors indicating responsiveness to radiation, and considers quality of life and functional outcomes for patients undergoing various rectal-preserving treatment pathways.

Chapter 8 contains the conclusions to this work.

Some of the work in this thesis has been published; a list of publications to date is found in Appendix 1.

Chapter 2: Materials and methods

This thesis is principally based on clinical data and tissue samples from patients who have undergone local excision of rectal cancer by transanal endoscopic microsurgery (TEM) in Oxford between 2007 and 2017. This chapter covers the major methods used; other specific methods are dealt with in the relevant chapter.

2.1 Approvals and funding

2.1.1 Projects

Pre-treatment molecular stratification and the histogenic origins of rectal cancer: This was sponsored by Oxford University and approved under the Research tissue bank ethics reference: 11/YH/0020 and IBD Cohort 09/H1204/30 under the title: Molecular analysis of rectal tumours to investigate lesion pathogenesis, radiation sensitivity and lymph node metastasis. The project used tissue samples of locally excised rectal cancers and clinical data from the TEM database. Samples were processed in Prof Leedham's lab in the Wellcome Centre for Cancer Genetics, with the support of Dr Hayley Davis and other lab team members.

An observational study to correlate the results of ploidy and stroma analysis with prognosis in early rectal cancer: IRAS ID: 195159, OUH R&D PID number: 11846. This was sponsored by OUH and approved by the West Midlands – South Birmingham Research Ethics Committee, REC reference: 16/WM/0443, approved 28/10/2016. Approval was also obtained from the Norwegian ethics board. HRA approval was granted on 9/1/2017, Trust Management Approval on 29/3/2017. A Material Transfer Agreement was signed between OUH and the Institute for Cancer Genetics and Informatics, Oslo 17/3/2017. This project used tissue samples and clinical data from the same source as above. The tissue samples were analysed at the Institute for Cancer Genetics in Oslo by Dr Hanne Askautrud and team.

Assessment of a 13-gene extracellular matrix signature for predicting radiosensitivity and outcome in rectal cancer: This was sponsored by Oxford University and accepted under the GI Illness biobank and IBD Cohort umbrella and approved under the Research tissue bank ethics reference: 16/YH/0247. Samples were processed in the Radiobiology Research Institute by Alex Gordon-Weeks.

Feasibility of sentinel lymph node biopsy in rectal cancer: IRAS ID: 155425, OUH R&D PID number: 11324. This study was sponsored by OUH and approved by the South Central – Oxford A Research Ethics Committee, REC reference: 15/SC/0317 on 1/7/2015. It was adopted onto the CRN portfolio and run by NIHR Direct Delivery Team in accordance with the current approved protocol, the principles of the Declaration of Helsinki, ICH Guidelines for Good Clinical Practice (CPMP/ICH/135/95) July 1996, and relevant regulations and standard operating procedures, and in conformity with the European Commission Medical Device Guidelines relating to the application of the EU Directives on Medical Devices and the Guide to European Medical Device Trials and BS EN ISO 14155. A Research Collaboration Agreement was signed between OUH, the University of Leeds and Endomagnetics on 2/9/2015. It was registered on clinicaltrial.gov (NCT02445456). Trust Management Approval (TMA) was granted on 16/12/2016. During the trial, two amendments became necessary. The first allowed for MRIs on all patients, not just the TEM group, and the use of intra-operative ICG dye for assessment of the lymphatic drainage in the radical surgery group; this received REC approval on 27/6/2017 and HRA approval on 17/7/2017. The second amendment became necessary when the supplier replaced the tracer with a new preparation which contains the same active ingredient in a different solute; this received REC approval on 27/6/2019, HRA approval on 24/7/2019 and TMA on 4/12/2019 following contract amendment. Regular monitoring was performed according to ICH GCP. OUH R&D conducted a review of the consent process in 2018 with a satisfactory outcome. Throughout the study information was regularly updated as required for NIHR, CRN, Clinicaltrials.gov website, CRUK database of trials and HTC website.

2.1.2 Funding received

BDRF research grant of £60,000 for project: Pre-treatment molecular stratification and the histogenic origins of rectal cancer, to address the ACPGBI Delphi question: What biomarkers affect the response to chemoradiotherapy for rectal cancer?

Occtopus research grant of £5,000 for project: An observational study to correlate the results of ploidy and stroma analysis with prognosis in early rectal cancer.

Occtopus research grant of £10,000 for project: Assessment of a 13-gene extracellular matrix signature for predicting radiosensitivity and outcome in rectal cancer.

NIHR Impact Acceleration Award of £5000 from NIHR Colorectal Therapies Healthcare Technology Cooperative, Leeds for project: Feasibility of sentinel lymph node biopsy in rectal cancer. Matched funding for consumables and loan of the research device (magnetic probe) was provided by Endomagnetics, Cambridge. This covers 40 vials of tracer at £250 each. An internal OUH research fund covered costs of the MRI scans.

2.2 Materials

2.2.1 Oxford TEM database

The TEM database prospectively records information on all patients having TEM surgery in Oxford and is regularly updated. Data collected includes demographics, pre-operative staging, prior polypectomy, neoadjuvant treatment, details of surgical procedure, complications, histopathology of the tumour, post-operative management, and outcome. Data on some histopathology variables such as tumour morphology, budding, width of invasive front and depth of invasion below the muscularis mucosa were inconsistently collected in the earlier years. It is worth noting that Oxford is a tertiary referral centre; many referred patients came from outside the region and were specifically exploring organ-preserving options.

2.2.2 TEM tissue samples

In accordance with the approved protocol and agreements, for all patients who had TEM for rectal cancer between 2007 and 2017 and who consented to use of tissue for ethically approved research, the operative fresh-frozen paraffin-embedded (FFPE) tissue blocks were retrieved by OCHRe. A pathologist selected two tissue blocks representative of the tumour where sample size and quality permitted. From one block, 16 5µm sections were cut using a microtome by the OCHRe team; 10 were mounted on uncharged slides and six on charged Superfrost Plus slides. The other block was transported to Oslo where sections were cut, then the blocks were returned to OCHRe.

2.2.3 Rectal cancer biobank

Additional fresh tissue samples were collected for a rectal cancer biobank and a database established to provide clinical details for the work described in Chapter 7. In accordance with the approved protocol and agreements, all patients with suspected rectal cancer attending the endoscopy department for surgical assessment of the tumour between April 2017 and April 2019 when team members were present were invited to participate in the biobank. For patients who consented, two biopsies were taken from the tumour and one biopsy from the normal rectal mucosa via the endoscope. Blood samples were also taken. At each subsequent visit to the endoscopy department, patients were invited to continue participation. If they consented, a further blood sample was taken as well as biopsies of any residual or recurrent rectal tumour.

All biopsy samples were placed in tubes containing RNAlater solution and stored in a -80C freezer until required for processing. Blood samples were collected in an EDTA tube and centrifuged within one hour then stored in a -80C freezer. The associated Rectal cancer biobank database prospectively records information on all participating patients and includes data on demographics, tumour details, management and outcome.

2.2.4 Patient questionnaire on quality of life

A patient questionnaire was used to assess functional outcomes and quality of life after rectal-preserving treatments. This is a composite of several validated questionnaires looking at general measures and specifically at bowel, urinary and sexual function (Table 2.1).

The EuroQol EQ-5D-3L comprises an index score based on five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression, and a visual analogue scale (EQ-VAS) showing the patient self-rated health[85].

TABLE 2.1: VALIDATED QUESTIONNAIRES CONTAINED IN THE COMPOSITE FORM

Questionnaire	Purpose
EuroQol EQ-5D-3L	Overall quality of life, 5 dimensions, 3 levels
EORTC QLQ-C30	General quality of life for cancer patients
EORTC QLQ-CR29	Colorectal supplement
LARS	Low anterior resection syndrome
Vaisey	Bowel incontinence
IPSS	Urinary function
IIEF-5	International index of erectile function, men only, 5 dimensions
MFSQ-9	McCoy female sexuality questionnaire, women only, 9 dimensions

* EORTC = European organisation for research and treatment of cancer.

The EORTC QLQ-C30 was developed to assess the quality of life in cancer patients[86]. It contains 30 items, which are tallied to give a global health and quality of life scale, five functional scales: physical, role, cognitive, emotional and social, and several symptom scales. The EORTC QLQ-CR29 was designed to evaluate colorectal cancer therapy from a patient's perspective[87] and comprises 29 items including ano-rectal, urinary and sexual functioning. Ano-rectal function was additionally assessed using the LARS and Vaizey scoring system. The LARS system comprises five items and gives a total score between 0 (no LARS) and 42 (major LARS)[88]. The Vaizey score[89] has seven items and gives a total between 0 (perfect continence) and 24 (totally incontinent).

The IPSS specifically assesses urinary function[90] and has been shown suitable for women[91]. It comprises seven questions on specific symptoms and an overall quality of life due to urinary

symptoms item. The overall IPSS score ranges from 0 (asymptomatic) to 35 (severely symptomatic). Male sexual function was assessed with the IIEF-5 score[92], which contains five items and gives a total from 5 (severe dysfunction) to 25 (no dysfunction). Female sexual function was assessed with the short MFSQ-9[93] which is composed of nine items assessing four dimensions, giving a total ranging from 9 (less function) to 63 (higher function).

2.2.5 National MDT survey on management of early rectal cancer

An electronic survey was prepared to assess the UK national perspective on management of early rectal cancer. This consisted of 10 questions with optional free text boxes on a Google Form, available at https://forms.gle/Rj6x1rHGF3nvhcP99 and Appendix 2. The survey was developed locally and tested on colorectal surgeons in Oxford and Odense, Denmark. The survey was kindly emailed to the nominated contact for all UK colorectal MDTs by the Pelican Cancer Foundation in September 2019 with a reminder in December 2019 and return cut-off of end-February 2020.

2.3 Methods

2.3.1 Clinical data analysis

2.3.1.1 TEM database analysis

A retrospective analysis was undertaken, including all patients with a diagnosis of invasive malignancy, either in the TEM specimen or in a pre-operative polypectomy or biopsy, over an 11-year period from 2007-2017. For specimens where no residual tumour was present at TEM, the pathological stage of the previous polypectomy specimen, or the MRI stage for tumours with complete response to neoadjuvant radiotherapy, was used. Size of tumour refers to the size of the invasive component.

2.3.1.2 Patient questionnaire on quality of life

Between April 2017 and April 2019 all patients attending Oxford for surveillance following rectal-preserving treatment for rectal cancer were asked to complete a quality of life and functional outcomes questionnaire at their follow-up visit. All were disease-free at the time of evaluation. Patients who had undergone radical surgery were not included.

For patients having composite treatment the time till questionnaire completion was calculated from the date of the last treatment. Incomplete questionnaires were included if most of the items had been answered. If the response was missing to an item, or one or more items that contributed to a summary score were missing, that item or score was not included in the analysis of that item or score.

2.3.1.3 Statistical analysis

Data were collated and descriptive statistics obtained in Excel (Microsoft). Data are presented as number and percentage, median and range or interquartile range (IQR), or mean and standard deviation (sd) as appropriate and stated in the relevant text. Differences between groups were tested for significance using χ^2 test for categorical data, or Fisher's exact test if the expected frequency in any cell was less than 5. Normally distributed continuous data was assessed using t-test. For non-parametric data the Mann-Whitney test was used to compare two groups and the Kruskal-Wallis test for more groups. Differences between proportions were tested using the binomial proportion test. Correlations were assessed using the coefficient of determination, R^2. ANCOVA was used to check for the effect of potential confounders on outcomes across groups. A p value ≤ 0.05 was considered statistically significant.

R statistical software (www.r-project.org) was used to undertake significance testing, uni- and multi-variable analysis, correlation, ANCOVA, linear, logistic and ordinal regression, propensity score matching, Cox proportional-hazards regression, Kaplan-Meier survival analysis and Receiver Operating Characteristic (ROC) curves. For uni- and multi-variable analyses, patients with missing information for a particular variable were not included in the analysis of that variable.

For survival analysis, patients were censored at the date of last known follow-up or death. Local recurrence was defined as pelvic or rectal recurrence. Local recurrence-free survival was calculated from the date of TEM to the date of detection of local recurrence or censoring. Disease-free survival (DFS) was calculated from the date of TEM to the date of detection of local or distant disease, or censoring. Overall survival (OS) was calculated from the date of TEM to date of death from any cause or censoring.

For assessing optimal cut-point on a ROC curve, the Youden index was used, which maximises the value of sensitivity + specificity – 1.

PREDICTED LOCAL RECURRENCE

For a subset of patients, the predicted local recurrence rate at 3 years was taken from a published table of predicted risk[14], and the summed risk compared with the observed rate. To enable comparison with the model the same methodology and inclusion and exclusion criteria were implemented for the relevant analyses, according to the information presented in the publication.

PROPENSITY SCORE MATCHING

Propensity score matching based on age, sex, date of surgery, tumour stage, size and height, lymphovascular invasion and resection margin status was used to identify a group followed by surveillance alone after TEM that was matched to a group who had adjuvant radiation then surveillance to compare outcomes.

2.3.2 Sample analysis

2.3.2.1 Histopathology gene panel testing

The Oxford histopathology department introduced molecular testing in 2013; initially this just involved testing for *KRAS* mutation, but since 2015 colorectal cancer samples are routinely tested for MSI status by immunostaining for the four DNA mismatch repair (MMR) markers and against a mutation panel of 50 genes shown in Table 2.2. This analysis is performed on macro-dissected FFPE tissue containing over 50% tumour. The next-generation sequencing (Ion Torrent PGM) assay uses a targeted hotspot strategy to identify >95% of mutations in genes in the panel, with a sensitivity of 5-10%.

TABLE 2.2: THE 50-GENE PANEL USED FOR ROUTINE TESTING OF COLORECTAL CANCER TISSUE SINCE 2015; ONLY MUTATIONS IN THE NINE GENES IN BOLD ARE ROUTINELY REPORTED.

BRAF	*FGFR1*	*CTNNB1*	*SMO*	*JAK3*	*EZH2*
KRAS	*ERBB2*	*CDKN2A*	*SMAD4*	*AKT1*	*GNA11*
NRAS	*MET*	*ABL1*	*VHL*	*KDR*	*GNAQ*
PDGFRA	*FGFR3*	*NOTCH1*	*NPM1*	*ALK*	*IDH2*
PIK3CA	*FLT3*	*ATM*	*MPL*	*JAK2*	*SRC*
KIT	*RB1*	*ERBB4*	*GNAS*	*MLH1*	*APC*
PTEN	*CSF1R*	*FGFR2*	*HNF1A*	*HRAS*	*CDH1*
EGFR	*RET*	*STK11*	*FBXW7*		
TP53	*SMARCB1*	*PTPN11*	*IDH1*		

Only the MSI status and mutations in the first nine genes are recorded on the histopathology report. Data on the additional 41 genes was obtained manually from separate files held in the histopathology department for samples where testing had been done.

2.3.2.2 Haematoxylin and Eosin staining

For each sample one paraffin section was stained with Haematoxylin and Eosin (H&E) for histological examination using the following protocol: sections were dewaxed in xylene for 5 minutes, then rehydrated through graded ethanols (100%, 90%, 70%) for 5 minutes each followed

by 2 minutes in tap water. Sections were then stained in Harris Haematoxylin for 30 seconds and washed in running water until the water ran clear. The slides were then briefly dipped into acid alcohol and left in water for 5 minutes. Sections were then stained in Eosin for 3 minutes, followed by a quick dip in water and then dehydrated through degraded alcohols for 2-5 minutes each, before mounting a coverslip with DPX.

2.3.2.3 DNN digital pathology

The H&E stained section for each sample was marked up to indicate the cancer and any adjacent polyp by Prof Simon Leedham. The slide was scanned on the Hamamatsu scanner at the Ludwig Institute for Cancer Research and scans transferred to pathologist Prof Viktor Koelzer in Zurich, Switzerland for digital analysis. Digital pathology was undertaken using a deep neural net algorithm (DNN) to classify and quantify tissue composition and cell level segmentation on the scanned slides[94]. Each area was segmented into square millimetres to be quantified. The DNN was trained on several thousand manually annotated tissue regions from the digital slide sets of S:CORT[95] and TCGA (The Cancer Genome Atlas)[96].

The slides were initially reviewed by the pathologist for quality control and regions of invasive cancer were annotated. After training, the DNN automatically segmented the cancer region into the following compartments and quantified the area in mm^2:

- Background (corresponds to whitespace, excluded from subsequent analysis)
- Necrosis (excluded from subsequent analysis)
- Epithelium (tumour area)
- Desmoplastic stroma (fibroblast-rich activated stroma, no immune cell infiltration)
- Inflamed stroma (lymphocyte-rich)
- Mucin (acellular mucin)

- Mesenchyme (non-neoplastic mesenchymal components of bowel wall, e.g. smooth muscle).

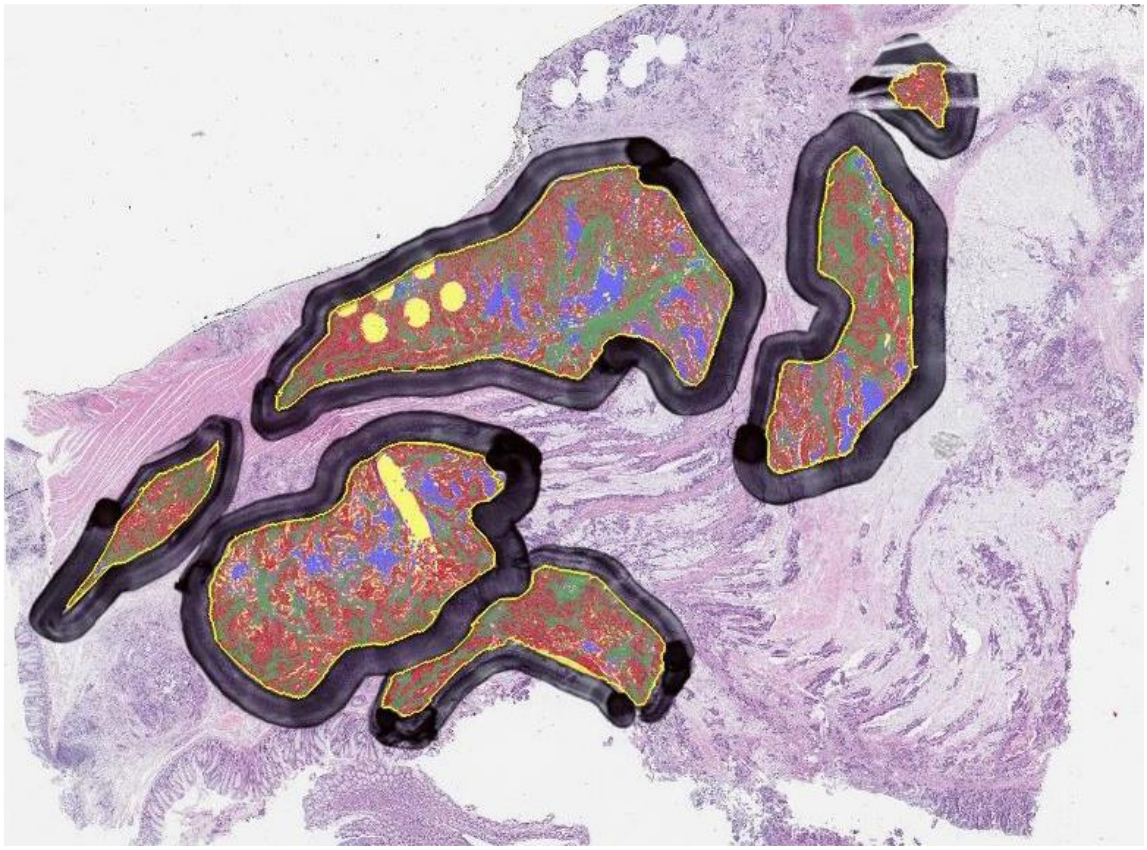


FIGURE 2.1: EXAMPLE OF A TUMOUR ANNOTATED USING A DEEP NEURAL NET ALGORITHM. TISSUE COMPARTMENTS: RED = EPITHELIUM; GREEN = DESMOPLASTIC STROMA; PURPLE = INFLAMED STROMA; BLUE = MUCIN; YELLOW = WHITESPACE; PINK = NECROSIS. IMAGE COURTESY OF V. KOELZER.

An example of a classified tumour is shown in Figure 2.1. Within each compartment the cells were segmented and counted. Cell density in each compartment was calculated as cell number divided by compartment area. Cell morphometry was also assessed, recording the average nuclear size, perimeter and haematoxylin staining intensity. These measurements are absolute values based on the segmentation of the H&E slide and provide a measure of cell morphometric features averaged across the millions of cells in a particular sample. This provides a good representation of the cellular morphometric measurements in the particular case. However, the standard error when making these measurements on a standard H&E slide, as used in this study and which tends to be thicker

and have less standardisation of the staining protocol, is higher than when using a purpose cut and stained haematoxylin-only slide. Relative difference in nuclear area compared to perimeter is calculated as the difference between the average area and average perimeter, divided by the average area of the nuclei.

2.3.2.4 Immunohistochemistry

One slide from each sample was stained for AXIN2 by Leticia Campo at the Ludwig Institute for Cancer Research. Automated staining was carried out with the Leica BOND-MAX autostainer (Leica, Microsystem) using the following conditions: antigen retrieval at 100°C for 20 min with Epitope Retrieval Solution 2 (AR9640, Leica Biosystems), primary antibody incubation with the AXIN2 antibody (Abcam, ab32197) at 1:2000 dilution or IgG control for 30mins and detection using the BOND™ Polymer Refine Detection System (DS9800, Leica Biosystems) as per manufacturer's instructions.

2.3.2.5 Stromal Fraction

This was undertaken at the Institute for Cancer Genetics and Informatics in Oslo by Dr Hanne Askautrud and team. A detailed methodology has been published[64]. A section from each sample was stained with H&E and scanned. A pathologist annotated the tumour area on the images using a software tool and the images were processed by several correction steps. Two masks were created, one for background and one for connective tissue. After further image processing the masks were combined to allow calculation of the tumour stromal fraction.

2.3.2.6 Chromosomal instability

This was also undertaken at the Institute for Cancer Genetics and Informatics, using a locally developed high throughput image-based system. Using an annotated H&E slide to identify the tumour area, tissue sections were treated enzymatically and washed to leave a suspension of nuclei which were spun onto a slide as a monolayer and stained with Feulgen's stain. A robotic microscope photographed each nucleus and the images were processed and categorised using a specifically developed Ploidy Work Station (PWS). This collates the images into galleries according to shape, size, chromatin content and chromatin texture, as shown in Figure 2.2. Only epithelial nuclei are considered and non-intact nuclei are discarded. If fewer than 300 valid nuclei are present the sample is excluded from further analysis.

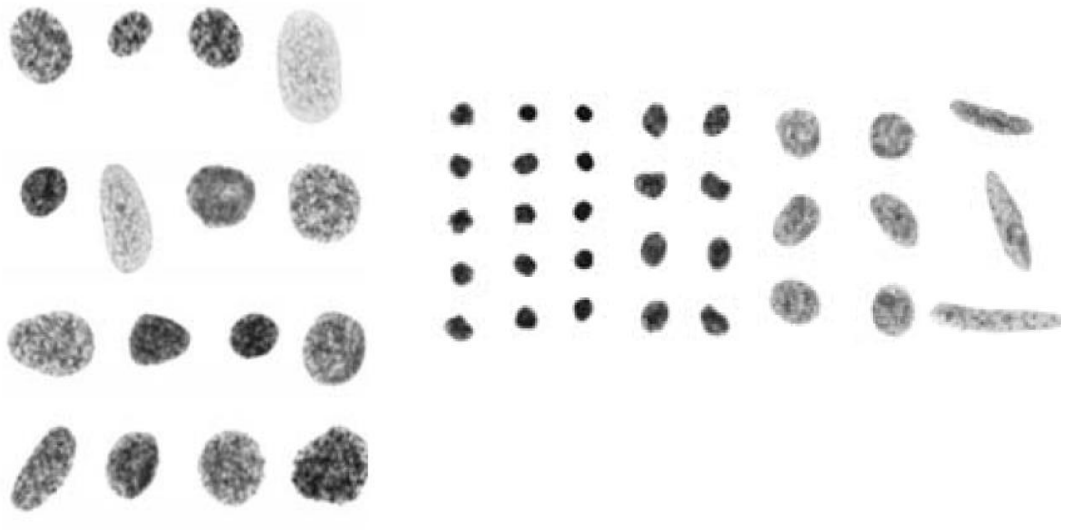


FIGURE 2.2: EXAMPLE OF DIGITISED NUCLEI IMAGES (LEFT) AND A CLASSIFICATION GALLERY OF NUCLEI (RIGHT). IMAGES COURTESY OF H ASKAUTRUD, ICGI

DNA PLOIDY

The optical density was measured and multipied by the area of each nucleus to give the integrated optical density; this was used to create a DNA ploidy histogram for each sample. These histograms were classified into diploid, aneuploid, tetraploid and polypoid. A detailed methodology[97] and classification criteria[98] have been published.

NUCLEOTYPING

The nuclear images are then further digitally processed to quantify the chromatin organisation. Each nuclear pixel is taken to be the centre of a subregion and the entropy, defined as disorder of the chromatin organisation, is calculated from the grey level at the central pixel and the variability in the grey level for the subregion. Multiple measurements are made for variously sized subregions around each pixel, and an average value is calculated for each sample. This is used to derive a score termed the chromatin value which describes the overall amount of chromatin disorder in the sample nuclei. A detailed methodology has been published[51].

2.3.3 Tissue sample processing

Five uncharged slides were deparaffinised by placing in xylene for 2 minutes, then rehydrated through graded ethanols (100%, 90%, 70%) for 1 minute each followed by 1 minute in nuclease-free water. Using the marked H&E slide as a guide, these slides were dissected using a 21-gauge needle to extract tumour tissue for RNA isolation. A further five uncharged slides were deparaffinised and dissected in the same way for DNA isolation. If polyp was also present in the sample, the carcinoma and adenoma regions were dissected separately so RNA and DNA could be extracted for each region.

2.3.3.1 RNA extraction

RNA was extracted from five slides for each sample using the Roche High Pure FFPET RNA isolation kit (v3, October 2012, modified protocol). The protocol was modified, omitting the DNase treatment. Following extraction, RNA was quantified spectrophotometrically using 1-2µl of extract on the NanoDrop (NanoDrop One/One, ThermoFisher). Using the NanoDrop measurement as a guide, up to 8µl of extract was then treated with Invitrogen Amplification Grade DNase treatment (ThermoFisher, catalogue number: 18068015) to remove DNA from the RNA extract. This DNAse

method was used in preference to that in the Roche isolation kit as there was some concern about the quantity and quality of RNA that could be obtained from the FFPE samples and preliminary investigation showed more RNA remained after the Invitrogen method. Following DNase treatment, the RNA sample was quantified again using the Qubit RNA HS assay kit and 2.0 Fluorometer (Invitrogen) to ensure an accurate measure. Plates were prepared for analysis using a standard amount of RNA in each well, diluted with RNase-free water where necessary. When not in use the RNA samples were stored in a -80°C freezer.

2.3.3.2 DNA extraction

DNA was extracted from five slides for each sample using the Roche High Pure FFPET DNA Isolation kit (v03, March 2015, modified protocol). Following extraction, DNA was quantified spectrophotometrically using 1-2µl of extract on the NanoDrop (NanoDrop One/One, ThermoFisher) and then using the Qubit dsDNA HS assay kit and 2.0 Fluorometer (Invitrogen) to ensure an accurate measure. When not in use the DNA samples were stored in a -20°C freezer.

2.3.3.3 RNA sequencing

The extracted RNA was submitted in five batches, with 51, 22, 18, 62 and 15 samples respectively, to the WHG Oxford Genomics Centre for 3' mRNA sequencing. The reference genome used was GRCh37.EBVB95-8wt.ERCC. Once sequenced, WHG Bioinformatics Core undertook preliminary processing of the raw data to produce gene count tables, quality control reports and tables of annotated results of differential gene expression for specified sample groups.

2.3.3.4 Other RNA processing

RNA was prepared in four batches for fusion analysis. Three batches were shipped to Genomics Birmingham for quantification of *RSPO2* and *RSPO3* gene fusions using a QIAseq Targeted RNAscan Custom Panel with sequencing on 1 MID Output 300 cycle flow cell (FC-404-2003). One batch was processed in Oxford by Sam Kleeman. Fusions were identified by a motif search.

Qualitative reverse transcription polymerase chain reaction (PCR) was done by Sam Kleeman to measure expression of *AXIN2* and *RSPO3* in all samples using a Fluidigm Biomark HD Real-Time PCR platform. The RNA was reverse transcribed into complementary DNA which was then pre-amplified using the Fluidigm Preamp Master Mix and run on a 192.24 IFC chip. Each reaction was performed in quadruplicate and the expression of each target was normalized to endogenous controls (*EEF1A1*, *ACTB* and *GAPDH*).

2.3.3.5 DNA processing

Two plates of DNA were prepared with 65ng DNA in 13µl and shipped to Genomics Birmingham for assessment using molecular inversion probe (MIP) targeted panel sequencing. This was to look for common functional mutations and recurrent genes fusions including the *RNF43* mutation. The 30 genes targeted are listed in Table 2.3.

TABLE 2.3: LIST OF 30 GENES ASSESSED USING MIP TARGETED PANEL SEQUENCING

ACVR2A	*BCL9L*	*FBXW7*	*MSH6*	*PTEN*	*SOX9*
APC	*BMPR2*	*GNAS*	*NRAS*	*RNF43*	*TCF7L2*
ARID1A	*BRAF*	*KRAS*	*PIK3CA*	*RPL22*	*TGIF1*
ATM	*CTNNB1*	*MLH1*	*POLD1*	*SMAD2*	*TP53*
B2M	*ELF3*	*MSH2*	*POLE*	*SMAD4*	*ZFP36L2*

2.3.3.6 RNA extraction from biobank samples

RNA extraction from the fresh frozen biobank samples was done by Alex Gordon-Weeks in the Radiobiology Research Institute. RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instruction manual.

2.3.3.7 Polymerase chain reaction analysis

Polymerase chain reaction (PCR) for the ECM signature study (Chapter 7) was performed by Alex Gordon-Weeks using the QuantStudio 5 Real-Time PCR system (ThermoFisher). SYBR Green I dye chemistry was used. Relative quantitation was done using the comparative CT (threshold cycle) calculation method. Concentrations were normalised to GAPDH and β-tubulin.

2.3.4 Bioinformatic analysis

The RNA sequencing results were annotated with Ensembl identifiers. For some analyses these required conversion to Entrez identifiers. This was initially done using the DAVID online tool[99], and the GeneCards website[100] for any that could not be found. Analysis was undertaken in R statistical software (www.r-project.org) using several packages; see the Appendix for details.

2.3.4.1 Signature scores

The ESTIMATE scores[101] for stromal and immune signature were calculated using the Estimate R package[102]. The package initially intersects a list of 10,412 common genes with the sample dataset. Each signature is based on 141 genes and is designed to measure the presence of infiltrating stromal and immune cell related genes and so infer the fraction of stromal and immune cells in the tumour sample. Combining these two scores gives the ESTIMATE score, and from this the R package calculates a measure of tumour purity.

An epithelial signature for each sample was obtained by creating a gene set of epithelial marker genes from Tan *et al.*[103]. Using the MultiGSEA[104] R package this gene set was used to obtain single sample GSEA (gene set enrichment analysis) epithelial scores for each sample. Three different methods were used to calculate the scores: ewm (eigen weighted mean), z score and ssgsea. These were inspected to determine the most appropriate, and the ssgsea method chosen for the epithelial signature.

A Wnt signalling score was also obtained for each sample using single sample GSEA and the GSVA[105] and MultiGSEA R packages. Preliminary analysis used curated Wnt pathway gene lists from the MSig database[106] Hallmark and C2 collections and the three different scoring methods listed above to determine the most appropriate method for this data. Again, the ssgsea method was chosen as the most suitable. The most appropriate gene set was selected by examining heatmap plots of the sample gene set scores aggregated by z score.

2.3.4.2 Molecular subtype classification

The R package CMScaller[107] was used to assign CMS categories to each tumour. This package uses the nearest template prediction method and incorporates Camera gene set analysis against 15 pre-selected CMS-informative gene sets. The analysis was done using 25,255 genes with Ensembl identifiers with false discovery rate (FDR) set at 0.05. For samples that were not classified at the initial run, a second run was performed using 14,233 genes where an Entrez identifier was available.

The R package CRISclassifier[108] was used to assign CRIS categories to each tumour, using both the nearest template predictor (NTP) and top scoring pairs (TSP) methods. The TSP method was selected as providing results closer to the expected distribution.

2.3.4.3 Differentially expressed genes

GENE ONTOLOGY

Using the list of differentially expressed genes between groups of interest at FRD <0.05, the most expressed gene ontologies (GO) were determined using the R Bioconductor packages Goseq and Goana. These packages use different gene identifiers, so both were used to ensure no discrepancy; both returned the same results. Gene length information was used to correct for possible bias. The hg19 build of the human genome was used as reference. In Goseq, two methods were used to calculate over- and under-expressed GO categories; the default Wallenius approximation and one using random sampling to generate a null distribution[109]. A correlation plot indicated that there was little difference between the two methods.

GENE SET ENRICHMENT ANALYSIS

Gene set enrichment analysis (GSEA) differs from GO enrichment analysis in considering all genes rather than just the differentially expressed genes[110]. The R package FGSEA[111] was used. The analysis was repeated using two distinct gene sets. First, the Hallmark gene set in the Molecular Signatures Database (MSigDB) v6.2[112] was used. Secondly, the Reactome gene sets were used[113]. Significantly enriched pathways were considered to be those with an adjusted p value (corrected for FDR) <0.01. The FGSEA package was also used to obtain collapsed pathways, to identify independent pathways that were enriched.

KEGG PATHWAY ANALYSIS

The R package Limma was used for KEGG analysis[114] (Kyoto Encyclopedia of Genes and Genomes) and ClusterProfiler[115] and PathView[116] packages were used to produce graphical outputs.

PROTEIN-PROTEIN INTERACTION NETWORK ANALYSIS

Protein-protein interaction (PPI) network analysis was done using the R String.bd package[117], and considered the differentially expressed genes. Most genes mapped to protein identifiers in the String database. Plots of interactions between proteins were obtained. Lists of significantly

enriched gene ontologies and KEGG pathways were also obtained to ensure consistency with the methodologies above. Significantly expressed protein domains among the 200-400 most expressed genes were assessed using the PFAM[118] and Interpro[119] protein domains.

Chapter 3: Sentinel lymph node detection using magnetic nanoparticles

3.1 Introduction and background

This chapter presents a clinical trial undertaken to assess the feasibility of a magnetic tracer in identifying lymph nodes for biopsy as a prognostic factor in rectal cancer. A number of people have contributed to this work; see the Acknowledgements for further details.

While local excision for early rectal cancer has less morbidity and mortality than radical surgery, the ongoing uncertainty about lymph node status commits the patients to a prolonged regime of close surveillance so any recurrence can be detected at an early stage. If the sentinel lymph node could be identified during the local excision and removed for histopathological analysis, some of this uncertainty would be removed. The additional information gained about nodal status would contribute to decisions about subsequent management: negative nodes would provide more confidence to patient and clinician about continuing a rectal-preserving strategy, although surveillance remains important as recurrence may be luminal as well as nodal, whereas nodes containing cancer cells would indicate the need for further treatment or closer surveillance.

3.1.1 Sentinel lymph nodes

If the lymphatic drainage from an organ follows a predictable path, and cancer cells from a tumour spread via the lymphatic system from the tumour to the nearest lymph node, and thence further afield, sampling of the first (sentinel) lymph node (SLN) that tumour cells reach provides information about how far the tumour has progressed. Sentinel lymph node biopsy (SLNB) has been used to good effect in breast cancer treatment[120]. Axillary node dissection, a procedure associated with significant morbidity, was standard practice in breast cancer management. In contrast, targeted removal of a few axillary lymph nodes is associated with less morbidity and is more likely to identify nodes containing cancer deposits. Analogously, removing all the mesorectal lymph nodes involves total mesorectal excision (TME), a major procedure with significant morbidity; this

is the current standard treatment for rectal cancer. If it were possible to identify and biopsy the SLN by less invasive means, there could be substantial advantage to rectal cancer patients, although there may be morbidity associated with sentinel node biopsy.

Several methods have been used to identify sentinel lymph nodes, including dyes, radioactive tracers and magnetic nanoparticles. The magnetic technique has the advantage that the tracer is non-toxic and non-radioactive. It has been successfully used to identify SLNs in breast cancer[121-123] and melanoma[124]. Magnetic nanoparticles have been used in rectal cancer to enhance the accuracy of MRI scans. Koh *et al.*[125] noted characteristic enhancement patterns in non-malignant and reactive nodes on MRI using ultrasmall particles of iron oxide (USPIO). They subsequently compared MRI following intravenous injection of nanoparticles with histopathology and found the pattern of particle enhancement on the MRI had the same sensitivity but higher specificity than morphology in identifying malignant nodes[126]. This may be relevant to future work investigating the role of nanoparticles in prognostic stratification.

3.1.2 Techniques for sentinel node identification in colorectal cancer

A review of sentinel node assessment in colorectal cancer in 2011[127] found 52 papers including over 800 rectal cancers. Overall, they found that SLN assessment had a low sensitivity regardless of method. Cahill *et al.*[128] used indocyanine green (ICG) intra-operatively with near infrared (NIR) laparoscopy and were able to identify lymphatic drainage patterns and sentinel nodes for colon cancer. Handgraaf *et al.*[129] injected ICG submucosally around the tumour via endoscope at the start of radical surgery in five patients with rectal cancer. Intraoperatively they identified a mean of two fluorescent nodes per patient. The TME specimen was also assessed ex-vivo at the end of surgery, a mean of 190 minutes after injection; a mean of four fluorescent nodes were identified per patient.

Recently, SLN assessment has been attempted during local excision of rectal cancer. Arezzo *et al.*[130] used the ICG method, injecting submucosally at the start of a TEM procedure in three patients. In

each they were able to obtain a lymphatic map using NIR though the rectal wall defect and identify and excise lymph nodes from the mesorectum, with pathological confirmation of lymph nodes in all specimens. Quaresima *et al.*[131] used radioactive technetium in a similar way in 57 patients, removing 'hot' mesorectal fat through the rectal wall defect. They routinely excised with a 1cm margin, then found residual radioactivity in 49% of patients, which was additionally excised. They obtained lymph nodes in 12/29 (41%) of those where no additional excision was done and 10/28 (36%) of those who had additional guided mesorectal excision, giving an overall lymph node retrieval rate of 39%.

Pouw *et al.*[132] compared a magnetic tracer with blue dye for identifying nodes ex-vivo following radical excision of colorectal tumours in 27 patients, including three rectal cancers, where standard histopathology did not reveal any nodal disease. At least one node was identified in 96% of tumours using magnetic nanoparticles and 89% using blue dye. The three nodes with the highest magnetic signal and the three blue nodes closest to the tumour were ultrastaged using serial sectioning and staining. This revealed isolated tumour cells in 22 nodes from 10 patients. The magnetic tracer identified 16/22 nodes and all ten patients. They note that the magnetic nanoparticle is of an ideal size, 50-150nm, to be retained in the sentinel node. Furthermore, it is much simpler to use than radioactive tracers. There are no reports yet of magnetic tracer use during TEM surgery.

3.1.3 Sentirect clinical trial

This trial was designed to assess whether magnetic nanoparticle tracer injection at endoscopy is safe and feasible in patients with rectal cancer, how the tracer is distributed in the mesorectum on MRI and by using the magnetic probe, and how these distributions compare with nodal findings at histopathology. For patients having local excision of rectal cancer it will also assess whether the tracer enables identification and safe removal of the sentinel node. This is a novel technique so detailed procedures will be developed as this feasibility study progresses. If successful, this study

has the potential to significantly improve surgical and functional outcomes for rectal cancer patients by allowing less radical surgery to be used where it is most appropriate and guiding the need for further treatment.

3.1.4 Objectives

The primary objective of the Sentirect trial is to establish the technical feasibility of using pre-operative endoscopic injection of a magnetic nanoparticle tracer in rectal cancer patients to enable (post-)operative assessment of SLN using the hand-held SentiMag magnetometer by:

1. Assessing the incidence of adverse or hypersensitivity reactions after tracer injection,
2. Assessing whether the tracer is still present and detectable at the injection site and in the lymph nodes at the time of surgery,
3. Determining the impact of tracer injection on subsequent surgery.

Secondary objectives are:

1. To radiologically evaluate tracer drainage patterns using high resolution MRI.
2. To measure the distribution and spread of the tracer using the SentiMag probe in mesorectal specimens after rectal cancer surgery.
3. To assess the accuracy of the tracer/ magnetometer system in identifying SLNs by comparing findings with standard histopathological assessment.
4. To establish the feasibility of SLNB during local excision of early rectal cancer using the tracer/ magnetometer system.

3.2 Methods

3.2.1 Trial design

There are two phases to the trial; an "ex-vivo" phase, which concentrates on determining how effectively the technique identifies lymph nodes in the mesorectum in patients undergoing TME; and an "in-vivo" phase, which assesses the use of the system in enabling intra-operative SLNB during local excision. Figure 3.1 shows the study schema.

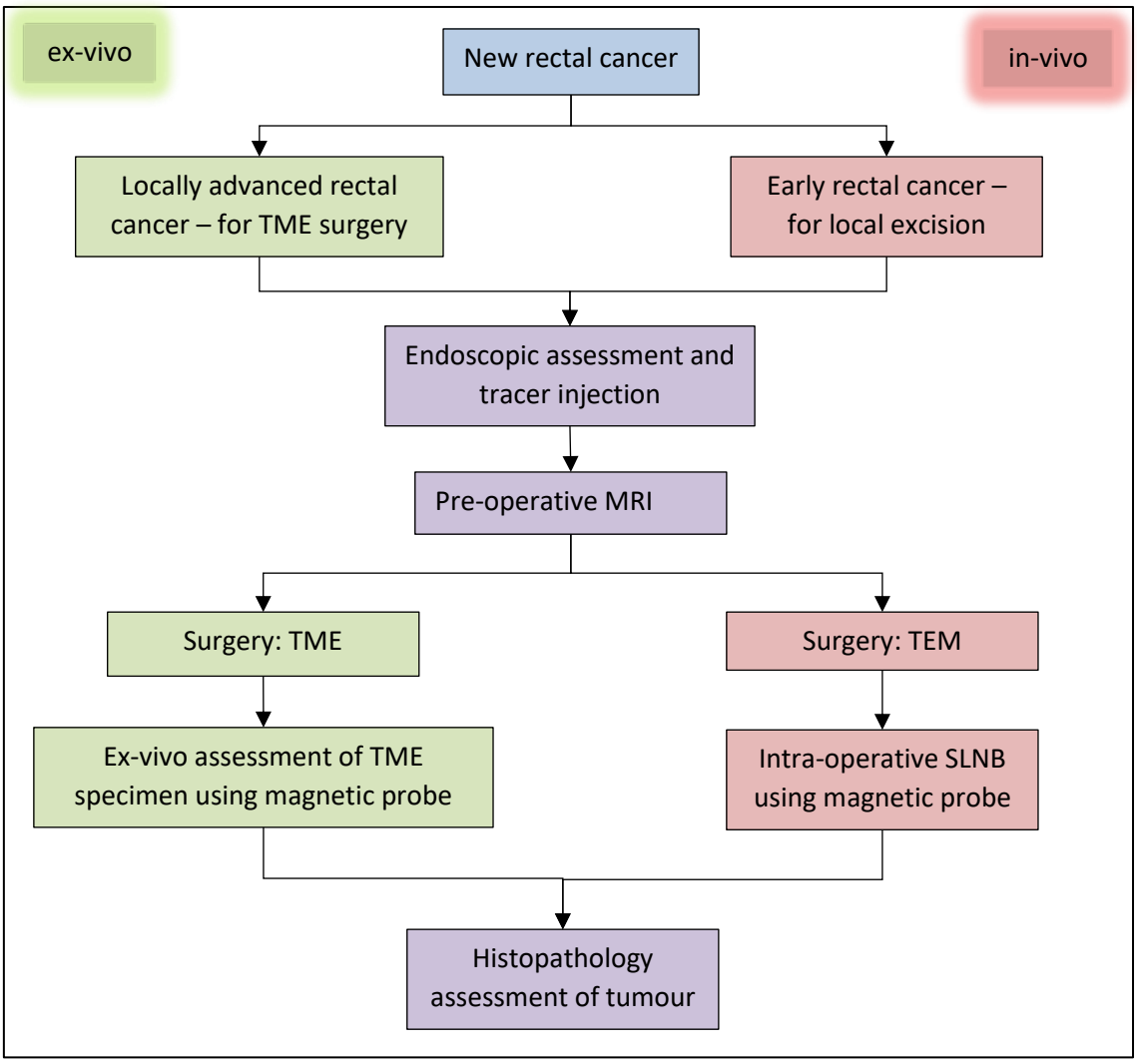


FIGURE 3.1: STUDY SCHEMA FOR THE SENTIRECT TRIAL

3.2.2 Patients

The trial participants are patients diagnosed with rectal cancer that is operable, with no symptoms of intestinal obstruction, and are scheduled for surgery at Oxford University Hospitals. They include both those scheduled for radical TME surgery as well as those scheduled for local excision by TEM. Participants must be willing and able to give informed consent (in English) for participation in the trial, and, in the Investigator's opinion, able and willing to comply with all trial requirements. Patients are excluded if they have a known intolerance or hypersensitivity to iron or dextran compounds, magnetic tracers, or SPIOs, or if they are unable to give consent or are deemed vulnerable.

The trial is designed to incur the minimum of extra intervention for participants. Standard practice includes a pre-operative clinic visit for discussion, visit to the endoscopy unit for assessment of the tumour by the surgeon and hospital admission for surgery. In addition to these standard procedures, there is one additional visit for an MRI scan. Where possible this is arranged on the same day as endoscopy or the day of surgery to minimise extra travel.

Number of Participants

The ex-vivo part of the study will recruit 20 patients due to have radical surgery for rectal cancer. The in-vivo part will recruit a further 20 patients with early rectal cancer scheduled for local excision. As this is a feasibility study, sample size calculation was not required.

3.2.3 Equipment

SentiMag system

This study uses Sienna+® and Magtrace tracers, which contains magnetised nanoparticles in a water or saline solution respectively, and a hand-held SentiMag® probe within its CE market intended

purpose for SLN mapping. This is a class IIa device, CE-approved in Europe and has been in use since February 2012. The manufacturer in Endomagnetics Limited, Cambridge.

The tracer is a dark brown aqueous suspension of organically coated superparamagnetic iron oxide particles, supplied in 2ml vials with a long shelf life. The storage and handling procedures are straightforward; there are no temperature issues. The vials are held in a secure storage area in the endoscopy department. The vial details and expiry date are checked prior to administration in accordance with standard procedures. Details of the batch and injection are recorded on the standard endoscopy documentation. Details of the injection are also recorded on the case report form (CRF). SentiMag includes a probe and portable base unit, which is on loan from the manufacturer (Figure 3.2). The probe is able to fit through the TEM scope without modification for intra-operative use. There are no safety issues. The equipment was assessed by the OUH clinical engineering department and passed the electrical safety test. The manufacturer is responsible for the quality control of the tracer and probe.



FIGURE 3.2: THE SENTIMAG SYSTEM, SHOWING PROBE AND HAND-HELD METER WITH VIALS OF TRACER.

Safety reporting is in accordance with the approved protocol. Expectedness of adverse reactions is determined according to the manufacturer's risk analysis report. Expected adverse reactions are uncommon but may include hypersensitivity reaction such as rash, itching, dizziness and light-headedness. If inadvertent injection into the vascular system occurs anaphylactic reaction is

possible. There may be brownish discolouration of the tissue around the injection site that may be noticeable at surgery.

Non-investigational medicinal product: Indocyanine green

ICG is a widely used tricarbocyanine dye with peak spectral absorption in the NIR range. Following injection, it can be visualised in the tissues using an NIR imaging system. ICG has a low molecular size so following injection into the bowel wall it is rapidly taken up by lymphatics and deposited in local lymph nodes. ICG contains sodium iodide; it is safe for general use but can cause allergic reactions in people with iodine sensitivity. ICG is supplied as a sterile water-soluble powder in 25mg vials. The vials are stored at room temperature. Prior to use the powder is dissolved in 10ml of sterile water. Once dissolved, ICG must be used within six hours.

3.2.4 Trial procedures

Training for investigators

At the start of the study, the investigators underwent methodology training and practiced the use of the hand-held SentiMag probe device under instruction from the manufacturer.

Techniques for injection, including dosage, volume and siting, and sentinel lymph node biopsy technique are to be standardized during this feasibility study as knowledge and experience is gained.

Recruitment

Suitable patients are identified at the weekly colorectal cancer multi-disciplinary team (MDT) meetings. Potential participants are approached by a research nurse to provide information about the trial and invite participation. Patients agreeing to participate are screened and recruited on their pre-operative visit to the endoscopy unit. The trial documents are attached in Appendix 3.

Informed consent

In the endoscopy unit a clinician is available to provide further information if needed. The research nurse obtains consent from patients agreeing to participate. Participants' GPs are informed of the study.

Endoscopic injection

The magnetic tracer (Sienna+ or Magtrace) is injected during pre-operative endoscopic assessment of the tumour; this is approximately five days before surgery. Up to 2ml of tracer is injected systematically in four quadrants into the submucosa around the rectal tumour. Patients are then monitored in the endoscopy recovery room for one hour to ensure no hypersensitivity or adverse reactions occur and provided with a contact telephone number in case of any delayed reaction or side-effects after discharge.

MRI scan of pelvis

After endoscopic injection of tracer, patients undergo an MRI scan of the pelvis to establish the drainage pattern of the tracer and mesorectal lymph node distribution; this is in addition to the earlier routine staging MRI scan. The MRI scans are reviewed by the trial radiologist and discussed with the investigators.

Rectal cancer surgery

Patients undergo rectal cancer surgery according to their clinical indication. Subsequent procedures depend on the trial phase.

EX-VIVO PHASE – MESORECTAL ASSESSMENT

During radical TME surgery for locally advanced rectal cancer, the "ex-vivo" group may receive an injection of ICG dye near the tumour to help in identifying lymphatic drainage patterns while confidence is developed with the SentiMag system. At the conclusion of surgery, the surgeon makes an assessment as to whether the magnetic tracer injection affected surgery, for example by causing discoloration or inflammation in the tissue planes.

IN-VIVO PHASE - SLNB

These patients with early rectal cancer undergo local excision by TEM. The tumour is removed by a standard TEM procedure. The surgeon then uses the hand-held SentiMag probe through the TEM scope to identify the SLN by seeking the site of maximal magnetic signal in the mesorectum through the rectal wall defect. Any area of peak magnetic activity in the mesorectum that can safely be excised is then removed. The defect in the rectal wall is sutured where possible in accordance with standard practice. The surgeon makes a note of any difficulties encountered during the attempted SLNB. The mesorectal biopsy is sent to the histopathology department along with the tumour for analysis.

Examination of the surgical specimen

In the ex-vivo phase, the surgical TME specimen is examined in the operating theatre following removal using the SentiMag probe. This is used to locate peaks of tracer activity and mark these with a suture as an indicator for the pathologist during subsequent histopathological assessment. An NIR imaging system is also used to assess the specimen to detect the distribution of ICG as an adjunct to identifying lymph nodes if ICG was injected intra-operatively. Identified sites are recorded on a diagram on the CRF.

Following examination of the specimen in the theatre, it is sent to the pathology department. There, a day or two later, the investigator and trial colorectal pathologist examine the specimen, again employing the SentiMag probe to mark the sites of highest magnetic signal with red paint prior to dissection (Figure 3.3).



FIGURE 3.3: EXAMPLE OF EXCISED SPECIMEN IN THE PATHOLOGY LABORATORY PRIOR TO DISSECTION, WITH RED INK INDICATING SITE OF MAXIMAL SIGNAL

The pathologist then examines the specimen to identify all lymph nodes present and whether they contain metastatic cancer deposits, according to standard practice, paying special attention to areas marked with a suture or red paint. The pathologist completes the relevant CRF as well as issuing a standard histopathology report. At the conclusion of the study the samples are held in the cellular pathology department, in line with standard practice for diagnostic surgical specimens.

For the in-vivo phase the locally excised tumour and any additionally excised lymph nodes are sent to the pathology department and undergo standard histopathological assessment.

Post-operative monitoring

Following surgery patients are admitted to the colorectal ward for routine post-operative care. During this time, they are monitored, and any adverse reactions or post-operative complications are recorded. The patients' participation in the study ceases at discharge from hospital.

3.2.5 Data analysis

Data collection

Data is recorded using paper CRFs and entered into an Excel spreadsheet. Data items collected include patient demographic and tumour details.

Outcome measures to address the primary objective are:

- Frequency of adverse or hypersensitivity reactions,
- Frequency of tracer being detectable at the tumour injection site and in the lymph nodes at the time of surgery,
- Surgeon's assessment of any operative difficulties encountered attributable to prior tracer injection.

Outcome measures to address the secondary objectives are:

- High-resolution MRI mapping of the drainage pattern of tracer,
- Mapping of tracer distribution in the mesorectum using the SentiMag probe (ex-vivo phase),
- Correlation between MRI findings and histopathological determination of nodal status,
- Correlation between histopathological findings and tracer uptake in the lymph nodes determined by SentiMag probe (ex-vivo phase),
- Proportion of TEM operations where the SLN could be identified during surgery using the SentiMag probe and successfully removed (in-vivo phase).

Statistical analysis

General methods are described in Chapter 2.

Impact of tracer injection on surgery was assessed by the surgeon's binary assessment as to whether surgery was more difficult than expected, and free text comments were analysed qualitatively.

The sentinel node is defined as the mesorectal lymph node with highest magnetic signal. A "positive" node is defined as having over 10% of the tracer activity of the site of initial injection. A "negative" node is defined as having less than 10% of the tracer activity of the initial injection site.

MRI and SentiMag mapping were qualitatively compared to establish a baseline of individual variability. The presence and location of lymph nodes in the mesorectum marked by SentiMag was compared with the histopathological findings for each specimen in the ex-vivo phase.

As this is a feasibility study, results were continuously evaluated, and adjustments made to technique as indicated. Any serious adverse event would prompt a review of the study and possible termination.

All patients who received the tracer injection were included in the analysis. All available data was used. Missing data was not imputed. Spurious data was re-checked, and if valid retained in the analysis.

Data management

Data were managed as set out in the approved protocol and in accordance with the NHS Code of Confidentiality and the Data Protection Act. Personal data will be stored and accessed by the investigators for less than 3 months. Research data will be stored for 10 years after the completion of the study. Participants are identified by a unique trial specific number in the trial database; no identifying data is held in the database. Access to the data is restricted to study team members.

3.3 Results

3.3.1 Recruitment

To date, 15 patients have been recruited into the trial (Figure 3.4). Recruitment was suspended for 12 months pending approval of the second amendment, which was granted at the end of 2019; the trial has now been extended till end-2020. Recruitment has been difficult from the start mainly due to the short timeline between a patient being referred with cancer and the endoscopy. Governance requires that patients are informed about the trial and provided with a patient information sheet at least 24 hours prior to recruitment, yet care must be taken not to approach patients before they

have been informed of the diagnosis of cancer. Furthermore, the trial has committed to minimising the impact on patients and not requiring extra hospital visits.

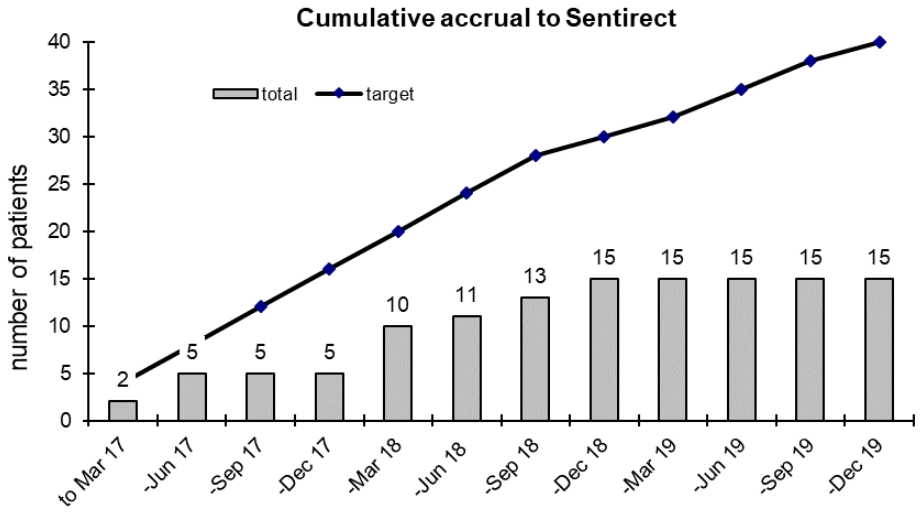


FIGURE 3.4: RECRUITMENT CHART FOR SENTIRECT TRIAL

Deviations from the protocol

Initially the ex-vivo phase patients did not have an MRI scan after tracer injection; this changed after the first amendment, so 11 had MRI scans. Two of the recruited patients did not proceed to surgery as after endoscopy a decision was made for neoadjuvant CRT; pathology specimens are not available for these. One recruited into the in-vivo phase had TME surgery. The protocol envisaged surgery five days after injection but in practice the interval was frequently longer due to logistical considerations.

3.3.2 Demographics

Table 3.1 shows details of the recruited patients and tumours; of the 15, 13 were recruited into the ex-vivo group. There were 12 males (80%) and three females, with median age 60 (range 32 – 85).

TABLE 3.1: DEMOGRAPHIC AND TUMOUR DETAILS FOR PATIENTS RECRUITED TO SENTIRECT. IDENTIFIERS BEGINNING WITH "A" INDICATE EX-VIVO GROUP, SCHEDULED FOR RADICAL SURGERY; "B" INDICATES IN-VIVO GROUP, PLANNED FOR LOCAL EXCISION.

Id	Age*	Sex	Preoperative staging	Prior treatment	Tumour diameter (cm)	Tumour height above anal margin (cm)	Tumour location
A1	61-70	M	mrT3 N0	Nil	5.3	6	posterior
A2	51-60	M	mrT2 N0	CRT	4	1.5	posterior
A3	<50	M	mrT2 N0	CRT	4	2.5	anterior
A4	51-60	F	mrT1 N0	TEM	1.8	8	posterior
A5	51-60	M	mrT3 N1	Nil	7	15	anterior
A6	61-70	F	mrT2 N0	Nil	3	15	posterior
A7	51-60	M	mrT3 N1	CRT	4	12	left lateral
A8	61-70	M	mrT3 N0	Nil	3	8	anterior
A9	61-70	M	mrT2 N0	TEM	2	12	left lateral
A10	51-60	F	mrT1 N0	Nil	2.5	7	posterior
A11	69	M	mrT3 N2	Nil	5	7	anterior
A12	<50	M	mrT1 N0	EMR	0.5	7	left lateral
A13	61-70	M	mrT2 N0	Nil	3	4	left lateral
B2	<50	M	mrT1 N0	Nil	2	10	anterior
B1	>70	M	mrT2 N0	Nil	3.5	12	posterior

* Age is presented in deciles to preserve data confidentiality.

Three patients had prior local excision of the tumour, two by TEM and one by polypectomy; they were recruited into the trial as they were scheduled for completion TME surgery. Another three patients had neoadjuvant radiotherapy prior to recruitment.

3.3.3 Endoscopy and tracer injection

All patients had successful injection of the Sienna tracer at endoscopy (Table 3.2). The amount of tracer injected varied over time as experience developed. The initial advice from the manufacturer was to use 2ml of tracer diluted up to 5ml in normal saline according to previous use in breast cancer; this proved too much volume for practical submucosal injection.

TABLE 3.2: ENDOSCOPY AND INJECTION DETAILS FOR RECRUITED PATIENTS

Id	Successful injection?	Tracer volume injected (ml)	Adverse effect?
A1	yes	2	no
A2	yes	0.4	no
A3	yes	0.5	no
A4	yes	1	no
A5	yes	1	no
A6	yes	1	no
A7	yes	1	no
A8	yes	1	no
A9	yes	1	no
A10	yes	1	no
A11	yes	1	no
A12	yes	1	no
A13	yes	1	no
B2	yes	0.5	no
B1	yes	2	no

The low recruitment, long time interval between each injection and variability in the tumours made it impractical to use a formal regime for testing each possible volume. After the initial few cases, the technique resolved upon as being the most practical and effective was injection of up to 1ml of tracer followed by 2ml of normal saline to chase the tracer through the injection tubing. Additionally, the initial advice to attempt four-quadrant submucosal injection around the tumour proved difficult to achieve so just one or two positions around the tumour were injected. Three patients complained of pain at the time of injection which quickly resolved. No other adverse effects were observed following injection.

3.3.4 MRI scan

MRI scans were done following injection in 11 patients (Table 3.3). The timing of the scan varied from the same day as injection to 36 days later, with a median of 11 days. Three patients had the scan on the day of injection. In these, tracer uptake was not noted in the tumour, but there was extraneous mucosal uptake. A similar pattern was seen in the scan at four days. Two of the three

same-day scans showed uptake only in the higher nodes. For patients where the scan was delayed by a week or more, tracer uptake was noted in the tumour but not extraneously in the mucosa. Uptake by nodes was noted in most of these.

TABLE 3.3: MRI SCAN DETAILS FOR RECRUITED PATIENTS

Id	Time from injection to MRI (days)	Tracer volume (ml)	Uptake by tumour?	Extraneous mucosal uptake?	Uptake by nodes?	Suspicious nodes?
A5	7	1	yes	no	yes	yes: 1
A6	36	1	yes	no	no	no
A7	28	1	yes	no	yes	yes
A8	14	1	yes	no	yes	no
A9	11	1	yes	no	yes	no
A10	15	1	yes	no	yes	no
A11	0	1	no	yes	higher only	no
A12	0	1	no	yes	higher only	yes: 1
A13	0	1	no	no	no	no
B2	4	0.5	no	yes	yes	no
B1	15	2	yes	no	yes	yes: 1

In one patient review of the MRI scan after tracer injection resulted in a change of staging as a node considered benign on the routine staging scan was now considered to show suspicious features. This may be due to suboptimal views on the earlier scan due to a hip replacement, with subsequent better visualisation of the 6mm (borderline enlarged), irregular node due to nanoparticle presence. The two patients who did not undergo surgery had repeated MRI scans after radiotherapy as part of a 'watch-and-wait' surveillance strategy. In both, the first follow-up MRI scan, done three months after injection, showed persisting nanoparticles in the rectal wall or mesorectum. The second scan, six months following injection, did not show residual artefact.

3.3.5 Surgery

Surgery was undertaken in 13 patients (Table 3.4). Most patients were recruited into the ex-vivo phase and had radical TME surgery. Two patients were recruited into the in-vivo phase and were

scheduled for local excision. One patient (B1) underwent local excision and attempted intra-operative localisation of lymph nodes using the SentiMag probe. However, there was a very strong but diffuse signal and it was not possible to localise any signal from a node that could be removed surgically. The second patient (B2) underwent attempted local excision, but at surgery the tumour was more advanced than anticipated so the procedure was abandoned. That patient returned a month later for radical surgery.

TABLE 3.4: SURGERY DETAILS FOR RECRUITED PATIENTS

Id	Time from injection to surgery (days)	Operation*	Operative difficulty due to tracer?	Duration of surgery (minutes)	Post-operative complication?	Hospital stay (days)
A1	5	LAR + TaTME	no	450	no	4
A2	5	APE	no	360	Small bowel obstruction	25
A3	6	APE	no	330	no	7
A4	5	LAR + TaTME	no	360	no	6
A5	32	LAR + TaTME	no	360	no	6
A6	39	LAR + TaTME	no	240	no	6
A7	41	LAR	no	330	ileus	32
A9	19	LAR	no	370	Anastomotic leak	31
A10	18	LAR	no	300	no	4
A12	33	LAR	no	360	Anastomotic leak	18
A13	32	LAR	no	300	no	5
B2	62	Lap LAR	no	360	no	8
B1	15	TAMIS	no	130	no	6

*LAR=low anterior resection, TaTME=transanal TME, APE=abdomino-perineal excision, lap=laparoscopic, TAMIS=transanal minimally invasive surgery.

The median time from tracer injection to surgery was 19 days (range 5 – 62). Only four (27%) patients had surgery within one week of the injection as foreseen in the protocol. The tracer was not found to hinder the surgical procedure in any patient. While a more systematic variation in time between injection and surgery would be ideal to assess the tracer effects, the operative

timings were determined by logistical considerations, and the unplanned variability fortuitously provided additional information compared to what the protocol has envisioned.

The median length of hospital stay after surgery was six days (range 4 – 32). Post-operative complications were noted in four patients, but none of these were attributed to tracer injection. Two of the patients who had complications (small bowel obstruction and ileus) had radiotherapy prior to surgery.

3.3.6 Pathology specimen

A surgical specimen was available from 13 patients, 12 radical procedures and one local excision (Table 3.5). The 12 specimens removed by TME were examined in the operating theatre using the SentiMag probe. The number of positive nodes identified with the probe varied from 0 to 2; identified nodes were marked with a suture.

TABLE 3.5: PATHOLOGY DETAILS OF REMOVED SPECIMENS

Id	Number of nodes identified using probe	pT stage	pN stage	Nodes removed: malignant/ total	Match between signal and node?
A1	1	2	0	0/33	no
A2	2	ypT3	1	1/20	Positive node 5mm above
A3	2	ypT2	1	1/14	no
A4	0	0	1	2/30	no
A5	2	3	0	0/36	no
A6	2	2	0	0/25	Large nodes nearby
A7	0	ypT3	2	11/38	no
A9	2	0	0	0/23	no
A10	0	2	0	0/26	no
A12	0	0	1	1/41	no
A13	0	2	0	0/27	no
B2	0	2	1	1/28	no
B1	0	2	x	n/a	n/a

The specimens were then sent to the pathology department for histopathological analysis. Some of these were re-examined using the SentiMag probe and marked with ink at the point of strongest signal. Overall, there was not a good correlation between the site of maximal signal and the presence of a lymph node. For those who had both MRI and histopathology available, two of three reported to have suspicious nodes on the MRI had cancer cells in the nodes on histopathology, and one of four who did not have suspicious nodes in the MRI had one involved node.

3.4 Discussion

This trial has struggled to recruit patients due to the inbuilt constraints in a rectal cancer patient's clinical management pathway and the requirements of good trial governance. Although recruitment was suspended for most of 2019, the trial is ongoing, and recruitment has been extended to the end of 2020 using the new tracer. The results above are therefore interim results.

3.4.1 Review of objectives

The primary objective of this trial was to establish the technical feasibility of pre-operative endoscopic tracer injection to enable SLN assessment at the time of surgery. It has been demonstrated that the injection technique is safe and achievable in routine clinical practice; no hypersensitivity or adverse effects related to tracer injection were observed. Furthermore, the tracer did not adversely impact subsequent surgery. So, the primary objective has been satisfied.

The secondary objectives have not been so successfully met. In one patient the MRI after injection caused a revision of staging, the tracer possibly compensating for suboptimal views on the routine scan due to a hip replacement, but more generally the post-injection MRI scan did not add value. Results to date have not shown strong correspondence between peak signal in the specimen and the presence of lymph nodes. The attempted intra-operative localisation of lymph nodes

demonstrated that the equipment could be used in this setting but, in this case, did not allow identification or removal of the SLN. This lack of success may reflect still-developing expertise with the technique, and the non-optimised time interval between injection and surgery. These are issues that will be addressed as the trial proceeds.

3.4.2 Lessons learnt concerning the SentiMag system

Results to date have established that the ideal injection technique in this setting is no more than 1ml of undiluted tracer, followed by 2ml of normal saline to chase the tracer through the tubing dead space. The injection is given in one or two submucosal sites around the tumour.

The results also allow a provisional determination of the optimal time interval between injection and MRI scan, and injection and surgery. The protocol envisaged that patients would have surgery within a week of injection, but practicalities meant this was adhered to only in a minority which fortuitously provided additional information about timings. Some tracer signal was detectable in the mesorectum in all cases at surgery, up to two months after injection. However, the tracer signal only positively identified a node in cases where the interval between injection and surgery was up to 39 days and not in those who had surgery beyond 40 days. This result therefore suggests that an interval of up to five weeks is acceptable for meaningful tracer detection.

The interval to MRI scan was also longer than envisaged in most patients. Although data is yet limited, preliminary results show a change in the pattern of tracer distribution after a week, with the mucosal tracer migrating from an extraneous location into the tumour. Furthermore, the early MRIs only showed tracer in the higher nodes whereas after a week more nodes in the mesorectum showed tracer uptake. This suggests that during the first week excess tracer is being flushed out, and after this time, tracer becomes more concentrated in the tumour and its draining nodes. This reflects the injection being given submucosally around the tumour rather than directly into the tumour. A more confident assessment could be made if patients had serial MRIs within the first

two weeks after injection. The current trial does not allow this but if a subsequent study were planned this would be worth examining more closely, with scans every four days between injection and surgery in the first 10 patients, to build a clear picture of tracer migration over time and individual variability. This finding also has an implication for the timing of surgery, as within the first week the tracer signal is most likely to represent 'overflow' at the injection site and flush out, with the tracer not yet localised in the tumour's draining nodes, making it difficult to localise sentinel nodes. Based on these findings, to get maximum value from the tracer, it can be tentatively suggested that MRI should be done at least 7-10 days after injection, and surgery within five weeks of injection. A subsequent study would need a more systematic approach to the timing of surgery, aiming for surgery at a fixed range of intervals between two and five weeks after injection to determine the optimal time for nodal localisation with the tracer.

3.4.3 Future work

The interim results to the trial have been somewhat disappointing and raise the question of whether the trial should continue. Although the results show the technique is feasible, they do not show it to be effective in identifying SLNs and guiding a biopsy. It is not yet clear whether this is due to the early stage of the learning curve in the use of the device, or whether it reflects an inherent inability of the device to sufficiently clearly localise nodes in the mesorectum. This could be due to the pattern of lymphatic drainage through the mesorectum in the deep pelvis, which may have different properties to the drainage in the more superficial situation of breast cancer and melanoma where the device has been used with more success. In both these situations, injection is given much closer to the time of surgery, within 24 hours or even intra-operatively[123,124] and the SLNB is done at a site remote from the primary tumour site, in the axilla, groin or neck. In contrast, the underlying aspiration of the Sentirect trial is to identify and remove mesorectal lymph nodes through the mucosal defect created by local excision. Such nodes must be within a short distance

of the primary tumour and with sufficient time elapsed since injection that the tracer has localised to these nearby draining nodes.

To optimise value as the trial proceeds it is important to incorporate what has been learnt already, and to focus on ensuring the best technique when attempting detection with the SentiMag probe; this will be achieved with the support of the manufacturer.

The vast benefit to the patient that could be realised by the ability to confidently remove and examine the node, both in providing reassurance and informing subsequent management, makes it worth ensuring that the potential of this technique is fully explored before drawing a conclusion. So, the trial will continue, building on the knowledge already gained, and if it shows more promise than hitherto will lead to a larger, more structured clinical trial.

Chapter 4: Clinical and histopathological prognostic factors

4.1 Introduction and background

This chapter analyses the Oxford TEM database, which has collected data on all patients having local excision of rectal cancer by transanal endoscopic microsurgery (TEM) since 2007. The chapter aims to establish a baseline regarding outcomes after this organ-preserving approach and the subsequent management pathways, and to identify potential prognostic factors.

4.1.1 Local recurrence after local excision

Local excision (LE) of rectal cancer shows higher rates of local recurrence, even for T1 tumours, than radical surgery. A review of the Norwegian Colorectal Cancer registry identified 543 patients with T1 cancer and 1,593 with T2, treated by TEM or TME without radiation[133]. The 5-year recurrence rate was higher after TEM: 14.5% v 1.4% for T1 and 11.4% v 4.4% for T2. They also noted that the overall survival was less in the TEM group, but when adjusted for age and sex, there was no excess mortality. A smaller Dutch study[134] found a 5-year local recurrence rate of 24% after LE compared with 0% after TME for T1 cancers. However, for a subset of tumours, those which were small, well-differentiated, with no lymphatic or vascular invasion, and which were completely excised, local recurrence rates equivalent to those after radical resection can be achieved[135]. The ACPGBI guidelines[17] for local excision are accordingly conservative, recommending complete, full-thickness local excision for T1sm1 tumours only.

The higher rate of recurrence is due to the mesorectum, which may harbour occult metastases, being left in situ. Radical resection after LE for 46 tumours staged as node-negative found involved lymph nodes in 20%[136]. Hase *et al.*[137] identified five features associated with lymph node metastases in colorectal cancer: tumour budding, poorly demarcated invasive front, moderately or poorly differentiated cancer cells at the invasive front, extension of tumour beyond sm1, and lymphatic invasion. No lymph nodes were involved if three or fewer were present; the rate was

33% if four were present, and 67% for all five. A systematic review[16] found three significant factors for lymph node metastases in T1 cancers: lymphatic invasion, submucosal invasion ≥1mm and poor differentiation.

Patients vary in their views about risk and the level of local recurrence risk they are willing to accept. Therefore, selecting appropriate patients for LE requires open and informed discussion with the patient, as well as careful pre-operative assessment of the tumour clinically, endoscopically, and with high-resolution MRI to assess tissue planes and lymph nodes. It is also necessary to consider whether LE will compromise later radical surgery should it become necessary, either because of unexpected adverse histopathological features in the locally excised tumour, or as a salvage procedure in the case of local recurrence.

4.1.2 Post-TEM management

Following LE, thorough histopathological assessment is essential to detect higher-risk tumours with an increased risk of positive lymph nodes, which may be present despite careful staging. If these features are found, ACPGBI guidelines[17] recommend completion radical surgery to reduce the risk of local recurrence. Other options to mitigate the risk of local recurrence are radiotherapy and close surveillance to allow early detection of recurrent disease. These may be a suitable consideration for patients who are unfit or prefer to avoid radical surgery.

4.1.2.1 Completion radical surgery

Completion surgery is defined as ‘surgery carried out after local excision to complete surgical treatment of the primary tumour. This is applied to patients with an inadequate or unclear resection margin following local excision, unfavourable pathology according to current standards and patients with a “low-risk” cancer who still wish to proceed to radical surgery after

counselling'[138]. This is distinguished from 'salvage surgery', a procedure with curative intent for local recurrence. However, while completion surgery reduces the risk of local recurrence, it introduces new risks: patients are exposed to all the adverse impacts of primary radical surgery, and indeed the operative risks may be higher as radical excision shortly after LE can be technically challenging, with a risk of perforation at the local excision site – 22% in a recent study[136]. This may yield a less-than-optimal quality mesorectal excision[138] and increase the APE rate compared with primary radical surgery[139]. There is a high incidence of post-operative complications[19,136]. A metanalysis[140] revealed residual cancer present in the completion specimen in 36% (73/202) and nodal metastases in 25% (53/212). Verseveld *et al.*[18] reported on all 144 completion procedures recorded in the Netherlands Cancer Registry from 2005-2015 and noted nodal disease in 13%. The metanalysis[140] found a local recurrence rate after completion surgery of 5.6% (18/321), comparable with rates of 3-5% after primary radical surgery[141,142], with an overall disease recurrence rate of 13% (35/262). Verseveld *et al.*'s 5-year relative survival (ratio of the observed survival rate to expected rates for general population) was 85% for T1, 90% for T2 and 73% for T3 cancers.

4.1.2.2 Adjuvant radiotherapy

Disease recurrence after LE is likely to be related to the presence of undiagnosed nodal disease, extramural vascular invasion, or implantation of cancer cells at the time of LE. Radiotherapy has long been used in combination with radical surgery for rectal cancer to reduce the local recurrence rate. The Swedish rectal cancer trial[143] found that pre-operative radiotherapy before TME decreased the 5-year local recurrence rate from 27% to 11% for all tumour stages combined. The same rationale led to the use of radiotherapy following LE when pathological features suggested that LE may not have been an adequate treatment. Radiotherapy can "sterilise" the excision bed and adjacent mesorectal lymph nodes and so reduce the risk of local recurrence.

Early results were not particularly promising, but with modern LE techniques and evolution in radiotherapy techniques, particularly the use of chemosensitising agents, efficacy has been improved with a reduction in adverse events. Over recent years, results have given more support to the concept. In a small UK study[144] 28 patients were offered adjuvant chemoradiation (CRT) following LE because of adverse histopathological findings; 18 accepted. The operative technique was transanal excision in the majority, with TEM in the remainder. At a median follow-up of 4 years there had been no local recurrence in the radiotherapy group but four instances of local recurrence among the 10 patients who declined. A Korean study[145] looked at 83 patients after LE. The standard adjuvant treatment was radiotherapy (50.4 Gy over 5 weeks) with concurrent chemotherapy. Three patients (3.6%) developed local recurrence, at a median follow-up of 5 years. A Japanese multi-centre trial[146] enrolled 61 eligible patients who had a transanal excision with high-risk features on histopathology; 57 agreed to CRT (45 Gy in 25 fractions) and four declined. In the CRT group 3.5% (2/57) developed local recurrence at a median follow-up of over 7 years while one of the four who declined CRT also developed local recurrence.

The Korean study[145] noted early proctitis (grade 2) in 11% of patients. The same number showed asymptomatic proctitis later at follow-up. In the Japanese study[146] of 57 patients, 26 early adverse effects were observed: only three were classified as grade 3. No late adverse effects were noted. This relatively mild risk profile contrasts with adverse events reported following CRT as a neoadjuvant treatment strategy. The Dutch CARTS study[147] which treated 55 patients with CRT (50 Gy in 25 fractions) prior to planned TEM recorded 39 grade 3-5 complications in 23 patients. There were two deaths during CRT, one due to sepsis and one attributed to arrhythmia. Furthermore, they noted a post-operative complication rate after TEM of 28% in patients who had neoadjuvant radiotherapy, higher than generally reported after primary treatment with TEM.

4.1.3 TEM after other treatment modalities

Some patients referred for TEM have already had treatment for rectal cancer, either neoadjuvant radiation prior to planned radical surgery or endoscopic polypectomy. In some of these, there is no evidence of residual cancer at TEM and the value of TEM is unclear.

4.1.3.1 Neoadjuvant radiotherapy

Neoadjuvant radiotherapy is common practice prior to standard radical surgery as it has been shown to improve outcomes. In more recent years, it has emerged as a potential organ-preserving strategy, as in some cases radiotherapy may induce a complete, or near complete, response, leaving no, or minimal, tumour detectable clinically or on imaging. Surgical resection in this case has often confirmed complete pathological response. However, after (near-)complete response the tumour may regrow or recur[148]. LE has a role in removing residual or recurrent tumour after radiotherapy in patients who are not fit for major surgery or wish for an organ-preserving approach.

4.1.3.2 Previous polypectomy

With the increase in screening endoscopy, the incidence of malignant polyps removed has increased[149]. The optimal management of these polyp cancers is unclear, particularly if the polyp was fragmented during removal and completeness of removal cannot be determined. Many can be considered cured by polypectomy alone, however there is a recognised small group that will develop extensive recurrent disease[150]. LE of the polyp stalk or scar has been advocated, but the benefit of this is unclear. The ACPGBI position statement on malignant polyps[151] recommends that surgical resection, either by further LE or standard surgery, of the affected area should be considered where the margin is <1mm.

Levic *et al.*[152] reported on 50 patients with unexpected malignancy in a polypectomy; 23 had subsequent surgery, 22 radical and one TEM, while 27 were observed. There were three post-operative complications including one death. Seven of 23 had residual tumour in the operative specimen. None in the operative group developed recurrence but in the observation group two developed local recurrence and two developed distant disease. Widmann *et al.*[153] raised issue with this paper as 55% of those in the observation group had positive or unassessable margins at polypectomy. De Jong *et al.*[154] undertook a systematic review of LE after incomplete T1 rectal polypectomy where the resection margin was ≤1mm or not assessable but high-risk histopathological features were not present. They found four studies covering 102 patients who had full-thickness TEM or transanal excision after polypectomy. Residual cancer was present in 9% and residual adenoma in 16% of TEM specimens; five patients subsequently had radical surgery because of T2 tumour or positive lymph node in the TEM specimen. At a median follow-up of 4 years one patient had developed local recurrence; distant recurrence was not mentioned. They therefore advocate further LE where the polyp is not clearly completely excised and conclude that incomplete excision alone does not imply an unfavourable prognosis so long as further LE confirms complete excision of the tumour. Junginger *et al.*[155] however noted three cases of local recurrence and two of distant metastases among 28 patients who had T1 cancer removed by polypectomy with 'unclear' margin but no other high-risk feature, then TEM with no residual tumour. They comment that absence of residual tumour at TEM does not guarantee cure, even for low-risk cancers.

4.1.4 Aims

This chapter considers the full set of TEM procedures and then certain subgroups to address some of the issues raised above. The final section asks about current UK practice in managing early rectal cancer. The aims are:

1) To determine rates of local recurrence and compare with earlier published rates
2) To identify risk factors for recurrence
3) To determine whether TEM should be done when there is no residual tumour following complete response to neoadjuvant radiation or polypectomy
4) To determine the outcomes after completion surgery
5) To assess the value of adjuvant radiation in reducing local recurrence after TEM
6) To understand current views of early rectal cancer management across the UK.

4.2 Methods

All methodology is described in Chapter 2: Materials and methods.

4.3 Results

4.3.1 Overall results

Over an 11-year period from January 2007 to December 2017 there were 229 TEMs undertaken for malignancy. These comprised 142 males (62%) and 87 females with a median age of 69 (range 31-94) years. A previous polypectomy had been performed in 61 patients, with TEM done to remove the residual stalk or scar; no residual malignancy was found in 34 of these. Pre-TEM neoadjuvant radiotherapy was used in 28 patients; in seven there was no residual cancer in the TEM specimen.

Tumours were staged as (y)pT1 in 131 (57%), (y)pT2 in 80 (35%) and (y)pT3 in 18 (8%). At TEM, 41 specimens were (y)pT0; in these cases staging was based on the previous polypectomy specimen or pre-radiotherapy MRI scan. Prior to TEM the tumour was considered benign in 45 (20%). Most TEMs were performed with curative intent, while 17 (7%) had TEM as compromise, aiming for cure but oncologically suboptimal, in patients with significant comorbidity or who declined radical surgery. Median follow-up was 4.0 years (range 0.1-11.7 years). For six patients who were referred

from out-of-region, no follow-up details were available after discharge. These six were omitted from the analysis. Figure 4.1 provides a flow chart of the study for the remaining 223 patients. The surveillance group includes the 28 patients who had pre-TEM radiation. Figure 4.2 shows the changes in pre- and post-TEM management pathways over the 11-year period.

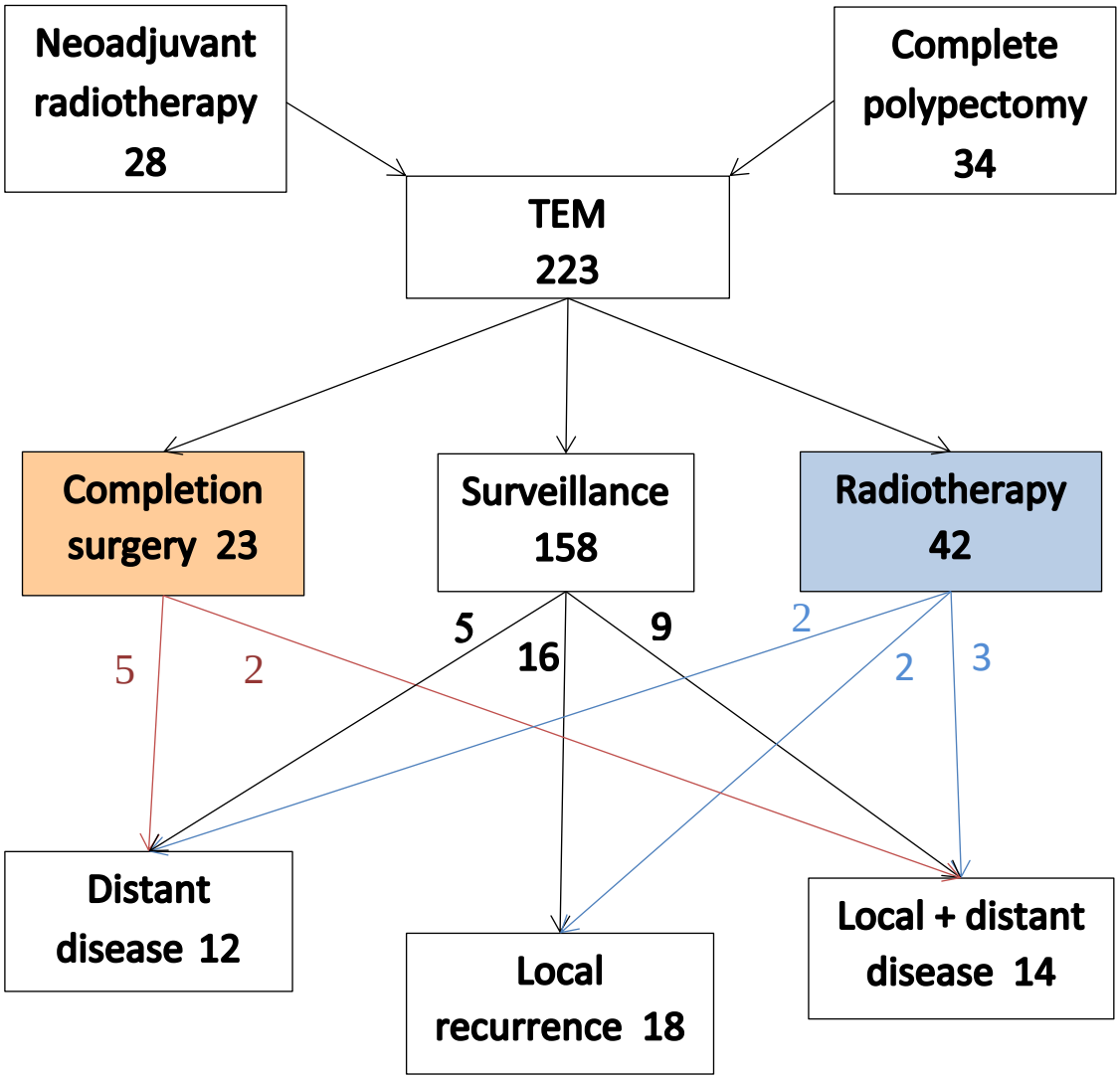


FIGURE 4.1: FLOW DIAGRAM FOR PATIENTS WITH RECTAL CANCER MANAGED BY TEM IN A SINGLE INSTITUTION OVER AN 11-YEAR PERIOD

The 28 patients who had pre-operative radiotherapy are excluded from the outcomes results presented in this chapter except where specifically mentioned, since the presenting tumour was

more advanced than in those undergoing primary TEM operation, leaving 195 patients for the main outcomes analysis.

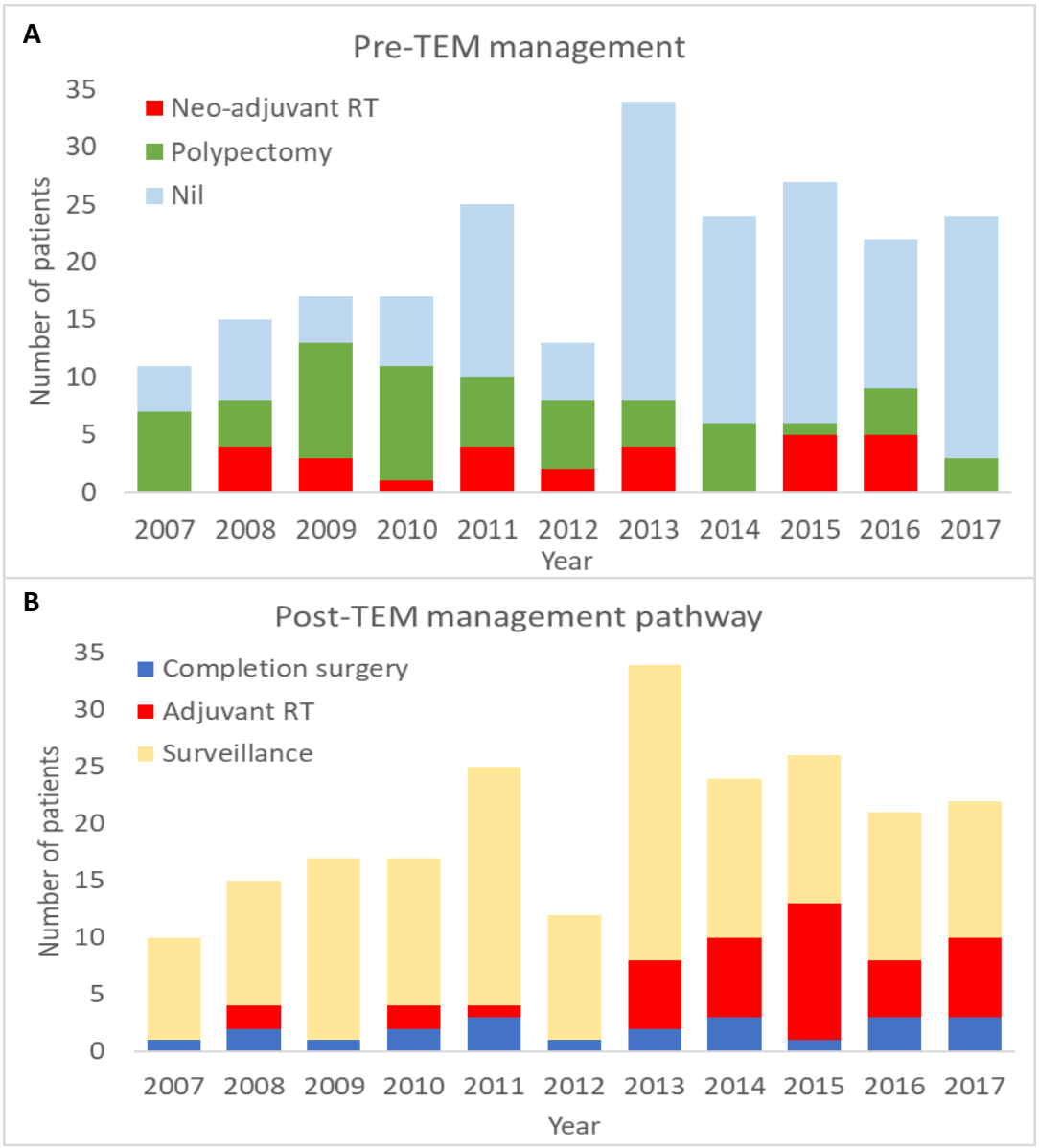


FIGURE 4.2: MANAGEMENT PATHWAYS OF PATIENTS UNDERGOING TEM FOR RECTAL CANCER. A. MANAGEMENT PRIOR TO TEM. B. MANAGEMENT AFTER TEM.

4.3.1.1 Surgery

Median operating time was 75 minutes (range 20 – 450). The operative technique was partial thickness (not including muscularis propria) in seven (3%), mixed partial and full thickness in 12 (5%) and full thickness (including muscularis propria) in 204 (91%). The peritoneum was opened,

intentionally or otherwise, during 21 procedures. Technical difficulties were noted in 22 cases; in five due to equipment problems, three due to tumour position, either high or low, six due to previous surgery or radiotherapy, and in eight no specific reason for the difficulty was noted. The median size of specimen removed was 18cm^2 (range 1 – 115). Median hospital stay after TEM was 2 days (range 0 – 71).

COMPLICATIONS

Three intra-operative complication of bleeding were recorded. Post-operative complications were recorded in 44 patients (19%), see Table 4.1. Sixteen patients (7%) were readmitted because of complications of the TEM procedure, eight of whom had prior radiotherapy. Further surgery for complications was required in 13 patients.

TABLE 4.1: POST-OPERATIVE COMPLICATIONS AFTER TEM SURGERY

Clavien-Dindo grade	Number	Type*
I Any deviation from post-op course not requiring significant intervention	9	7 fever 1 urinary retention 1 pain
II Requiring pharmacological intervention	13	7 bleeding 5 pelvic infection 1 chest infection 1 neuropathy
IIIa Intervention not under GA	9	9 TEM site dehiscence, managed by endoscopy
IIIb Intervention under GA	12	1 laparotomy for bleeding 2 EUA for bleeding 2 colostomy for leak 1 colostomy for faecal incontinence 3 (multiple) EUAs for stricture 2 EUAs for rectal abscess 1 I&D for perianal abscess
IVa Single organ failure	1	1 renal failure
IVb Multiorgan failure	0	
V Death	0	

* More than one type II occurred in one patient

4.3.1.2 Pathology

Histopathological details are shown in Table 4.2. Depth of invasion beyond muscularis mucosa, width of invasive front and tumour budding were only consistently recorded for the later years of the study period. The overall positive resection margin rate was 24% but this varied strongly with tumour stage: 12% for pT1, 34% for pT2 and 61% for pT3.

TABLE 4.2: HISTOPATHOLOGICAL FEATURES OF 229 RECTAL CANCERS REMOVED BY TEM

Tumour details	
Number of patients	229
Tumour stage of original tumour	
pT1*	131 (57%)
pT1 sm1	26 (11%)
pT1 sm2	32 (14%)
pT1 sm3	62 (27%)
pT2	80 (35%)
pT3	18 (8%)
Tumour diameter (cm)	
≤3.0	182 (79%)
3-5	38 (17%)
>5	9 (4%)
Differentiation	
Well differentiated	20 (9%)
Moderately differentiated	194 (85%)
Poorly differentiated	15 (7%)
Lympho-vascular invasion	79 (34%)
Resection margin positive	54 (24%)
Positive at deep margin	52
Positive at lateral margin	9
Resection margin <1mm	15
Resection margin involved	39
Depth of invasion beyond muscularis mucosa (mm), median (range), n=91	3.0 (0.2 – 9.0)
Width of invasive front (mm), median (range), n=105	12 (1.3 – 66)
Tumour budding, n=100	63 (63%)

*Kikuchi level was not available for 11 tumours

Of the 16 pT1 tumours with a positive resection margin, the deep margin was the positive site in 15 and the radial margin in one. In only four was tumour present at the deep margin; in the remaining 11, cancer cells were present within 1mm of the margin, at a median of 0.6mm (range 0.1-0.8mm). Of the 16, one was sm1, two were sm2 and 12 were sm3; for one the Kikuchi stage in not recorded. In five, the pre-operative diagnosis was benign. In one, the operative technique was

recorded as partial thickness, and in four as compound full and partial thickness; the remainder were described as full thickness excision.

4.3.1.3 Post-TEM management pathway

Completion surgery and/or adjuvant radiotherapy was considered in patients where the TEM specimen showed adverse features, i.e. one or more of the following: pT1sm3 or higher, poor differentiation, lympho-vascular invasion, mucinous, tumour budding and positive resection margins. Several patients elected not to proceed with either completion surgery or radiotherapy, in some cases because the low tumour site would have necessitated a permanent colostomy. A few further patients were not fit for radical surgery. All patients who did not undergo completion surgery were enrolled in a surveillance programme; those who had adjuvant radiotherapy were enrolled following completion of radiotherapy. The surveillance programme involves flexible sigmoidoscopy, pelvic MRI and CEA measurement every 3-4 months for the first 2 years, then 6 monthly till 5 years. CT chest-abdomen-pelvis is performed yearly up to 3 years. Any abnormalities prompt CT-PET scanning. Figure 4.2B shows the changing trends in management pathway over time and Table 4.3 details demographic and histopathology details for the different pathways.

COMPLETION SURGERY

Completion radical surgery was undertaken in 22 cases because of adverse histological factors and in another two because of complications either from TEM or subsequent radiotherapy. These cases are considered in more detail in Section 4.3.4.

ADJUVANT RADIOTHERAPY

Adjuvant radiotherapy was undertaken in 42 patients, mostly in the later years of the study. This starts at least six weeks following local excision, after complete healing of the TEM site had been confirmed endoscopically. Significant abnormalities such as sinus formation are given additional time to heal. The precise treatment regime may vary according to individual patient characteristics. The most common regime is 45 Gy radiation in 25 fractions daily for five weeks with oral

Capecitabine. If more limited treatment is indicated, 25 Gy in five fractions without chemotherapy was used. The irradiated fields are generally the same as standard fields for rectal cancer, comprising the TEM site, mesorectum and lateral pelvic walls. These cases are considered in more detail in section 4.3.5.

TABLE 4.3: DEMOGRAPHIC AND HISTOPATHOLOGY DETAILS ACCORDING TO POST-TEM MANAGEMENT PATHWAY

	Completion surgery	Adjuvant radiotherapy	Surveillance alone*	p value⁺
Number of patients	22	42	130	
Sex M:F	12(55%):10	27(64%):15	82(63%):48	0.72
Age at TEM, median (range)	66 (52-82)	68 (40 – 90)	70 (31 – 94)	0.36
Tumour stage				<0.00001*
pT1	7 (32%)	14 (33%)	102 (78%)	
sm1	0	0	22	
sm2	1	2	27	
sm3	6	14	33	
pT2	12 (55%)	21 (50%)	26 (20%)	
pT3	3 (14%)	7 (17%)	2 (2%)	
Tumour position				0.63
Anterior	5 (23%)	9 (21%)	22 (17%)	
Posterior	8 (36%)	14 (33%)	60 (46%)	
Lateral	9 (41%)	19 (45%)	48 (37%)	
Distance from anal verge				0.30
≤6 cm	10 (45%)	26 (62%)	64 (49%)	
>6 cm	12 (55%)	16 (38%)	66 (51%)	
Tumour diameter (cm)				0.0024*
≤3.0	13 (59%)	28 (67%)	114 (88%)	
3.1-5.0	8 (36%)	12 (29%)	12 (9%)	
>5.0	1 (5%)	2 (5%)	4 (3%)	
Resection margin positive	8 (36%)	18 (43%)	17 (13%)	<0.00001*
Lympho-vascular invasion	12 (55%)	26 (62%)	31 (24%)	<0.00001*
Differentiation				0.036*
Well differentiated	0	2 (5%)	16 (12%)	
Moderately differentiated	18 (82%)	38 (91%)	108 (83%)	
Poorly differentiated	4 (18%)	2 (5%)	6 (5%)	
Local recurrence	2 (9%)	5 (12%)	16 (12%)	0.91
Distant metastases	7 (32%)	5 (12%)	6 (5%)	0.00021*
Follow-up (years), median (range)	3.0 (0.3-9.2)	3.6 (0.1-10.1)	4.8 (0.2-11.7)	0.0042*

*excludes patients who had pre-operative radiation, ⁺ Chi-square or Fisher's exact test for categorical, Kruskal-Wallis for continuous variables

4.3.1.4 Local recurrence

After excluding patients who had pre-operative radiotherapy and where no follow-up was available, 195 patients remained. Local recurrence developed in 23 (11.8%) patients. In 15, local recurrence was isolated and was diagnosed at a median 23 months (range 3 – 112) following TEM. Eight patients developed local recurrence and distant disease; in four the local recurrence occurred first, being diagnosed a median of 9 months (range 4 – 11) after TEM, and the distant disease was detected a median of 21 months (range 13 – 34) after local recurrence occurred. In four patients, local recurrence was detected at the same time or after distant disease. The raw incidence by tumour stage was 7.3% (9/123) for T1, 18% (11/60) for T2 and 25% (3/12) for T3 tumours. Kaplan-Meier estimates of local recurrence risk are shown in Table 4.4, both by T stage of initial tumour and by post-operative management pathway. Figure 4.3 shows local recurrence-free survival curves. T stage had a significant impact of local recurrence-free survival, but post-TEM management pathway did not.

TABLE 4.4: ESTIMATED RISK OF LOCAL RECURRENCE AFTER TEM BY TUMOUR STAGE AND POST-TEM MANAGEMENT PATHWAY WITH 95% CONFIDENCE INTERVAL

	Number at risk	Estimated LR risk (95% CI)	Number at risk	Estimated LR risk (95% CI)	Number at risk	Estimated LR risk (95% CI)
	Tumour stage					
	pT1		**pT2**		**pT3**	
1 year	114	2.5% (0-5.2)	55	5.1% (0-11)	12	8.3% (0-23)
2 yrs	101	5.3% (1.1-9.3)	45	10.5% (2.2-18)	10	8.3% (0-23)
3 yrs	81	6.4% (1.7-11)	36	13.0% (3.4-22)	7	17.5% (0-37)
5 yrs	48	6.4% (1.7-11)	20	24.4% (10-37)	1	38.1% (0-67)
10 yrs	4	19.2% (0-37)	1	24.4% (10-37)		
	Post-TEM management pathway					
	Surveillance		**Completion surgery**		**Adjuvant radiotherapy**	
1 year	119	4.7% (1.0-8.3)	22	4.5% (0-13)	40	0% (0)
2 yrs	104	8.0% (3.1-13)	19	4.5% (0-13)	33	5.5% (0-13)
3 yrs	88	10.1% (4.5-15)	10	9.6% (0-21)	26	5.5% (0-13)
5 yrs	56	12.3% (6.0-18)	4	9.6% (0-21)	9	20.4% (1.4-36)
10 yrs	4	23.0% (3.7-38)			1	20.4% (1.4-36)

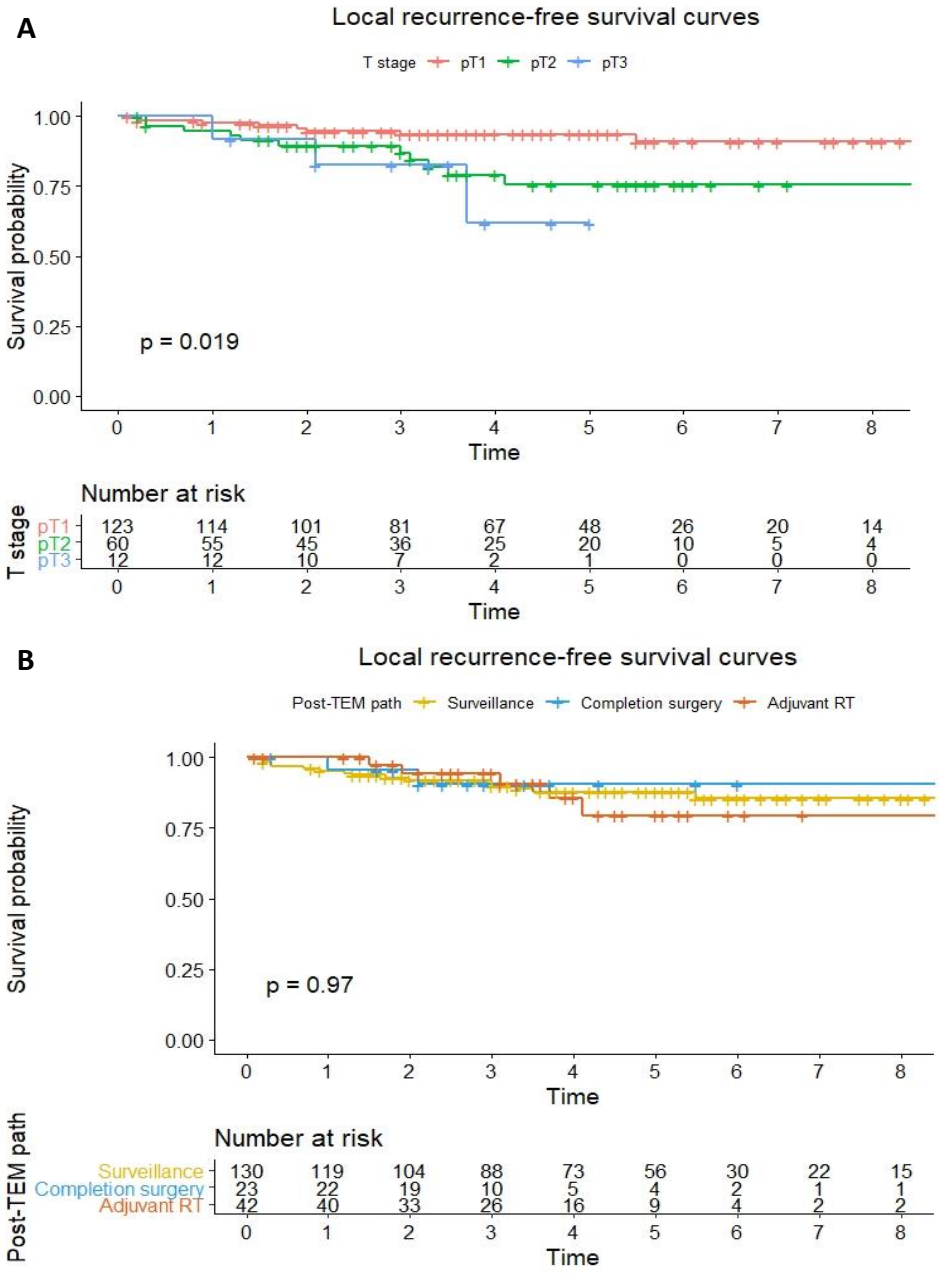


FIGURE 4.3: LOCAL RECURRENCE-FREE SURVIVAL CURVES AFTER TEM. A. ACCORDING TO TUMOUR STAGE. B. ACCORDING TO POST-TEM MANAGEMENT PATHWAY.

Univariate analysis (Table 4.5) showed a significant effect ($p<0.05$) on local recurrence for treatment intention (cure vs compromise), tumour stage and size, and neural, vascular and lymphovascular invasion. Tumour budding may also be significant as local recurrence was noted in 0/33 patients without budding and 4/58 (7%) with budding, but the event incidence was too small for assessment.

TABLE 4.5: UNIVARIATE ANALYSIS OF CLINICAL AND HISTOPATHOLOGICAL FEATURES FOR LOCAL RECURRENCE

Univariate analysis for local recurrence	No. of patients	No. of events	Hazard ratio	p value
Age	195	23	1.03 (0.99,1.08)	0.13
Sex				
F	74	7	1	
M	121	16	1.35 (0.56, 3.29)	0.51
Treatment intention				
Compromise	10	4	1	
Cure	185	19	0.11 (0.04, 0.34)	0.00010*
Pre-operative diagnosis				
Benign	42	2	1	
Malignant	153	21	2.77 (0.65, 11.84)	0.17
Position of tumour				
Anterior	37	3	1	
Lateral	75	9	1.52 (0.41, 5.61)	0.53
Posterior	83	11	1.71 (0.48, 6.12)	0.41
Type of lesion				
Flat	50	7	1	
Polypoid	118	13	0.93 (0.37, 2.34)	0.88
Ulcerated	27	3	0.82 (0.21, 3.17)	0.77
Distance from anal verge (cm)**	182	23	0.99 (0.88, 1.11)	0.82
Tumour mobility				
Mobile	167	20	1	
Fixed	28	3	0.88 (0.26, 2.95)	0.83
Operative technique				
Full-thickness excision	179	21	1	
Partial thickness	16	2	1.19 (0.28, 5.09)	0.82
Duration of surgery (min)	195	23	0.99 (0.99, 1.01)	0.64
Operative complication				
No	169	20	1	
Yes	26	3	0.85 (0.25, 2.89)	0.80
Hospital stay (days)	195	23	1.03 (0.98, 1.07)	0.27
Tumour diameter (cm)	195	23	1.30 (1.04, 1.61)	0.019*
Tumour stage				
pT1	123	9	1	
pT2	60	11	2.78 (1.15, 6.71)	0.023*
pT3	12	3	4.23 (1.13, 15.83)	0.032*
Tumour and Kikuchi stage				
pT1 sm1/2	60	1	1	
pT1 sm3	63	8	10.09 (1.26, 80.8)	0.029*
pT2	60	11	13.99 (1.80,108.5)	0.012*
pT3	12	3	21.50 (2.21,209.1)	0.0082*
Grade				
Well/mod differentiated	183	22	1	
Poorly differentiated	12	1	0.95 (0.13, 7.09)	0.96
Lymphatic invasion				
No	138	13	1	
Yes	57	10	2.02 (0.88, 4.61)	0.10
Vascular invasion				

No	154	15	1	
Yes	41	8	2.37 (1.00, 5.60)	0.05
Lymphovascular invasion				
No	125	10	1	
Yes	70	13	2.60 (1.14, 5.94)	0.023*
Neural invasion				
No	183	18	1	
Yes	12	5	5.07 (1.87, 13.75)	0.0014*
TEM resection margin				
R0	152	16	1	
R1	43	7	1.86 (0.76, 4.55)	0.17
Depth of invasion beyond muscularis mucosa (mm)**	80	5	0.98 (0.64, 1.50)	0.92
Width of invasive front (mm)**	94	7	1.02 (0.95, 1.10)	0.59
Post-TEM management pathway				
Surveillance alone	130	16	1	
Completion surgery	23	2	0.88 (0.20, 3.85)	0.86
Adjuvant radiotherapy	42	5	1.08 (0.40, 2.97)	0.88

**data not available for some cases

TEM was recorded as being a compromise, rather than cure, procedure in 10 patients and this was the single most significant factor on univariate analysis. For these 10, the median age was 83.5 (range 63-92) and median ASA grade 3 (range 2-3), while half the tumours were T1 and less than 2.5 cm diameter. This suggests that the decision for a compromise procedure was generally based on patient, rather than tumour, factors. Furthermore, there was a higher rate of positive resection margin for these (40%), implying less aggressive surgery. These 10 were excluded from the multivariable analysis.

A multivariable model was constructed for the 185 patients where the treatment intention was curative (Table 4.6). In this model tumour stage and neural invasion were significant, and lymphovascular invasion and post-TEM radiotherapy did not quite reach significance. In the multivariable model the only factors that remained significant were tumour stage and neural invasion, while post-TEM adjuvant radiotherapy almost reached significance and had a protective effect.

TABLE 4.6: MULTIVARIABLE ANALYSIS OF CLINICAL AND HISTOPATHOLOGY FEATURES FOR LOCAL RECURRENCE, FOR 185 CASES WHERE PROCEDURE INTENTION WAS CURATIVE.

Risk factor for local recurrence	Hazard ratio (95% CI)	p value
Tumour and Kikuchi stage		
pT1 sm1/2	1	
pT1 sm3	7.18 (0.86, 60)	0.069
pT2	10.08 (1.20, 85)	0.034*
pT3	15.18 (1.28, 181)	0.031*
Neural invasion	3.95 (1.25, 12)	0.020*
Lymphovascular invasion	2.37 (0.88, 6.4)	0.087
Adjuvant radiotherapy	0.34 (0.10, 1.1)	0.080

COMPARISON WITH PUBLISHED MODEL

Subgroup analysis was undertaken to enable direct comparison with a published model[14]. Local recurrence rates were estimated for 172 patients managed post-TEM by either surveillance or adjuvant radiotherapy. In this study 24% (42/172) of these had adjuvant radiotherapy, similar to the 23% (83/361) in the published study. Table 4.7 shows the expected rates according to the published model, and the observed rates in this study; the present results are consistently lower.

TABLE 4.7: COMPARISON OF OBSERVED LOCAL RECURRENCE RATE AND EXPECTED RATE ACCORDING TO A PUBLISHED MODEL[14]

		Estimated LR risk (95% CI)		
		Tumour stage		
		pT1	**pT2**	**pT3**
2 years	Expected	9.5%	23.7%	47%
	Observed	5.6% (1.1-9.8)	13.3% (2.8-22.6)	0%
3 years	Expected	12.9%	27.1%	na
	Observed	6.8% (1.6-11.6)	16.2% (4.3-26.6)	0%
5 years	Expected	18.6%	29.3%	na
	Observed	6.8% (1.6-11.6)	29.4% (12.4-43.1)	25.0% (0-57.4)

Estimates of local recurrence were also obtained for the group of patients younger than 80 and with well- and moderately differentiated tumours, by tumour stage, size and LVI status. There were 145 patients with 14 instances of local recurrence. Kaplan-Meier estimates of local recurrence at 3 years along with model-predicted values are shown in Table 4.8. Due to low numbers in some categories, useful estimates could not be determined for all groups, especially for tumours larger

than 4cm. The present series shows substantially lower rates for most categories, with the largest differences for the lower stage tumours.

TABLE 4.8: COMPARISON OF ESTIMATED 3-YEAR LOCAL RECURRENCE RATES IN THE PRESENT SERIES AND PUBLISHED MODEL PREDICTIONS, FOR WELL AND MODERATELY DIFFERENTIATED TUMOURS IN PATIENTS UNDER 80 YEARS[14]

			Estimated local recurrence rate at 3 years post-TEM			
			Maximum tumour diameter (cm)			
Tumour stage	**LVI**		**≤1**	**1.1–2**	**2.1–3**	**3.1–4**
pT1 sm1	No	Expected* Observed	3.0 0	3.6 0	4.4 0	5.4 0
pT1 sm1	Yes	Expected Observed	5.2 0	6.4 0	7.7 0	9.4 0
pT1 sm2-3	No	Expected Observed	10.5 0	12.7 10.3	15.3 11.1	18.5 0
pT1 sm2-3	Yes	Expected Observed	17.8 0	21.4 25.0	25.5 18.7	30.3 0
pT2	No	Expected Observed	9.8 0	11.9 0	14.3 20.0	17.3 0
pT2	Yes	Expected Observed	16.7 na	20.0 0	23.9 14.3	28.5 0
pT3	No	Expected Observed	19.7 na	23.6 0	28.0 na	33.2 0
pT3	Yes	Expected Observed	32.2 na	37.9 na	44.1 0	51.0 0

*Expected refers to the published figure; observed refers to the present study

Compared with the published series[14], fewer patients in the present series were thought to have a benign tumour prior to TEM (20% vs 38%), fewer had surgery with compromise rather than curative intent (7% vs 28%), and fewer had a less-than-full thickness excision (9% vs 21%), suggesting more thorough pre-operative assessment of the tumour, and a more aggressive approach to surgery. However, the rate of positive resection margin was not lower in the present series (24% vs 17%).

SALVAGE SURGERY

Salvage surgery was used to treat 14 of the patients who developed local recurrence; three of these had radiotherapy prior to the TEM procedure and a further two had had adjuvant radiotherapy. This group comprised 11 (79%) males and had median age 69 (range 49-86). The initial tumour was

pT1 in six (42%), pT2 in seven (50%) and pT3 in one (7%) although in three patients there was no tumour in the TEM specimen, two following prior polypectomy and one after neoadjuvant CRT. All the initial tumours were moderately differentiated and all but one were smaller than 3cm diameter; half showed lymphovascular invasion and in three (21%) the initial resection margin was positive. The median time from TEM to development of local recurrence was 17 months (range 3-112).

Some had been offered, but declined, completion surgery because of adverse features, and one had not been fit for completion surgery. The nine who had not previously had radiation were treated with CRT, with or without additional chemotherapy, for recurrence prior to proceeding to salvage surgery. Salvage surgery was undertaken a median of 2.1 years (range 0.4-10.5) following TEM. Some patients were out-of-region and limited information about the procedure and subsequent outcome was available.

Salvage involved a low anterior resection in five, abdomino-perineal excision in four, pelvic exenteration in four (one posterior only), and one Hartmann's procedure. Histology of the salvage specimen is available for ten patients. Of these, one had no residual tumour following CRT and three were ypT0 but had positive nodes. Of the residual tumours, three each were ypT3 and ypT4. The resection margin was involved in four, three at the recurrent tumour site and one at the nodal recurrence site, giving a positive margin rate of 4/10 (40%).

Following salvage surgery, one patient, aged 83, died of cardiac failure within 90 days. One patient developed further local recurrence and died three months after salvage surgery. A further three patients developed distant disease after salvage; one of these developed a lung metastasis 12 months after salvage, successfully treated by multiple lung ablations; the other two developed liver metastases, one with additional lung and nodal disease; both died, at 7 and 18 months post-salvage.

At last follow-up, three patients had died of disease (0.6, 1.5 and 2.7 years after salvage), two had died of other causes (0.2 and 2.8 years after salvage) and nine were alive with no evidence of

disease at a median of 4.0 years (range 0.4-9.5) after salvage surgery. In four of these the salvage procedure involved laparoscopic anterior resection with ileostomy that was subsequently reversed, while five had APR or exenteration, and had a permanent stoma.

Kaplan-Meier estimates of overall survival after salvage surgery are shown in Table 4.9, with estimates both from the date of TEM procedure and from date of salvage surgery. Estimates were also derived for the group that initially had TEM for pT1-2 tumour, to enable comparison with published results. The present results are not inconsistent with rates of 100%, 75% and 50% in a published series of 27 patients[15]. A recent study of survival for T1-2 tumours from the US National Cancer Database[156] found a 5-year survival rate of 81% after primary radical surgery. While this survival is higher than the 5-year survival after salvage surgery here – 62%, when survival is measured from initial TEM procedure, the values are much closer – 84% in the present series.

TABLE 4.9: ESTIMATED OVERALL SURVIVAL FOR PATIENTS UNDERGOING SALVAGE SURGERY FOR LOCAL RECURRENCE AFTER TEM, SHOWING SURVIVAL FROM SALVAGE PROCEDURE AND FROM TEM, FOR ALL CASES AND FOR THOSE WITH INITIAL PT1-2 STAGE TUMOUR

	Overall survival after salvage surgery		**Overall survival since TEM surgery**	
	Number at risk	Estimated survival	Number at risk	Estimated survival
	All salvage operations (n=14)			
At 1 year	10	84.4 (66.6-100)	14	100 (100-100)
At 2 years	8	75.0 (53.9-100)	13	92.9 (80.3-100)
At 3 years	6	56.3 (33.5-94.6)	12	85.7 (69.2-100)
At 5 years	2	56.3 (33.5-94.6)	9	77.9 (70.6-100)
At 10 years	0		2	53.4 (29.2-97.7)
	Salvage after initial pT1-2 TEM (n=13)			
At 1 year	10	92.3 (78.9-100)	13	100 (100-100)
At 2 years	8	82.1 (62.1-100)	13	100 (100-100)
At 3 years	6	61.5 (37.8-100)	12	92.3 (78.9-100)
At 5 years	2	61.5 (37.8-100)	9	83.9 (65.7-100)
At 10 years	0		2	57.5 (32.0-100)

4.3.1.5 Distant metastases

Distant disease developed in 18 patients, eight of whom also developed local recurrence. The local recurrence developed after the distant disease in four, while in four local recurrence preceded the distant disease. Of the 18 with distant disease, five had metastases only in the lungs, six in the liver

only, and the other seven had multiple sites involved, also including bone, brain, peritoneum and abdominal wall. Three of the four patients with peritoneal and abdominal wall disease had had completion surgery. For distant metastasis, univariate analysis showed a significant effect ($p<0.05$) for tumour stage and size, neural invasion, positive resection margin and post-operative management pathway; significant variables are shown in Table 4.10. For the most part, these are the same factors that were significant for local recurrence with the exception of lymphovascular invasion.

TABLE 4.10: UNIVARIATE ANALYSIS OF CLINICAL AND HISTOPATHOLOGY FEATURES FOR DISTANT METASTASES

Univariate analysis for distant metastases	No. of patients	No. of events	Hazard ratio (95% CI)	p value
Tumour diameter (cm)	195	18	1.41 (1.15, 1.72)	0.00082*
Tumour stage				
pT1	123	5	1	
pT2	60	8	3.89 (1.25, 12.05)	0.019*
pT3	12	5	14.19 (3.98,50.63)	0.000044*
Lymphatic invasion				
No	138	12	1	
Yes	57	6	1.25 (0.47, 3.32)	0.66
Vascular invasion				
No	154	14	1	
Yes	41	4	1.15 (0.38, 3.51)	0.81
Lymphovascular invasion				
No	125	10	1	
Yes	70	8	1.52 (0.60, 3.85)	0.38
Neural invasion				
No	183	15	1	
Yes	12	3	3.48 (1.00, 12.10)	0.049*
TEM resection margin				
R0	152	8	1	
R1	43	10	6.30 (2.39, 16.60)	0.00020*
Post-TEM management pathway				
Surveillance alone	130	6	1	
Completion surgery	23	7	9.99 (3.23, 30.92)	0.000065*
Adjuvant radiotherapy	42	5	3.06 (0.93, 10.09)	0.067

On multivariable analysis only three variables were significant: male sex, positive resection margin and post-TEM completion surgery were associated with a significantly higher incidence of distant metastases in the multivariable model (Table 4.11).

TABLE 4.11: MULTIVARIABLE ANALYSIS OF CLINICAL AND HISTOPATHOLOGY FEATURES FOR DISTANT METASTASES.

Risk factor for distant metastases	Hazard ratio (95% CI)	p value
Male sex	4.90 (1.46, 16.43)	0.01
Positive resection margin	8.85 (3.17, 24.66)	0.000031
Post-TEM completion surgery	8.88 (3.15, 25.04)	0.000037

4.3.1.6 Survival

DISEASE-FREE SURVIVAL

A total of 33 patients (16.9%) developed disease recurrence; 15 isolated local recurrence, 10 distant disease without local recurrence, and eight with both local and distant recurrence. Kaplan-Meier estimates of disease recurrence risk are shown in Table 4.12, both by T stage of initial tumour and by post-TEM management pathway. Disease-free survival is shown in Figure 4.4A & B, also by T stage and post-TEM management; both factors had a significant impact.

TABLE 4.12: ESTIMATED RISK OF DISEASE RECURRENCE AFTER TEM BY TUMOUR STAGE AND POST-TEM MANAGEMENT PATHWAY

	Number at risk	Estimated disease recurrence risk	Number at risk	Estimated disease recurrence risk	Number at risk	Estimated disease recurrence risk
	Tumour stage					
	pT1		**pT2**		**pT3**	
1 yr	112	4.2% (0.5-7.7)	52	10.3% (2.1-18)	10	25.0% (0-46)
2 yrs	99	6.9% (2.2-12)	41	17.6% (7.0-27)	7	34.4% (0.3-57)
3 yrs	80	8.1% (2.8-13)	34	22.1% (10-33)	6	34.4% (0.3-57)
5 yrs	48	8.1% (2.8-13)	19	32.4% (17-45)	1	50.8% (0.5-76)
10 yrs	3	36.5% (0-62)	1	32.4% (17-45)		
	Post-TEM management pathway					
	Surveillance		**Completion surgery**		**Adjuvant radiotherapy**	
1 yr	118	5.5% (1.4-9.3)	19	18.2% (0.4-33)	37	7.5% (0-15)
2 yrs	102	9.6% (4.3-15)	14	28.1% (6.1-45)	31	10.4% (0.2-20)
3 yrs	88	11.7% (5.7-17)	7	34.7% (9.5-53)	25	10.4% (0.2-20)
5 yrs	56	13.8% (7.2-20)	3	34.7% (9.5-53)	9	23.0% (5.5-37)
10 yrs	3	39.5% (0.9-63)			1	23.0% (5.5-37)

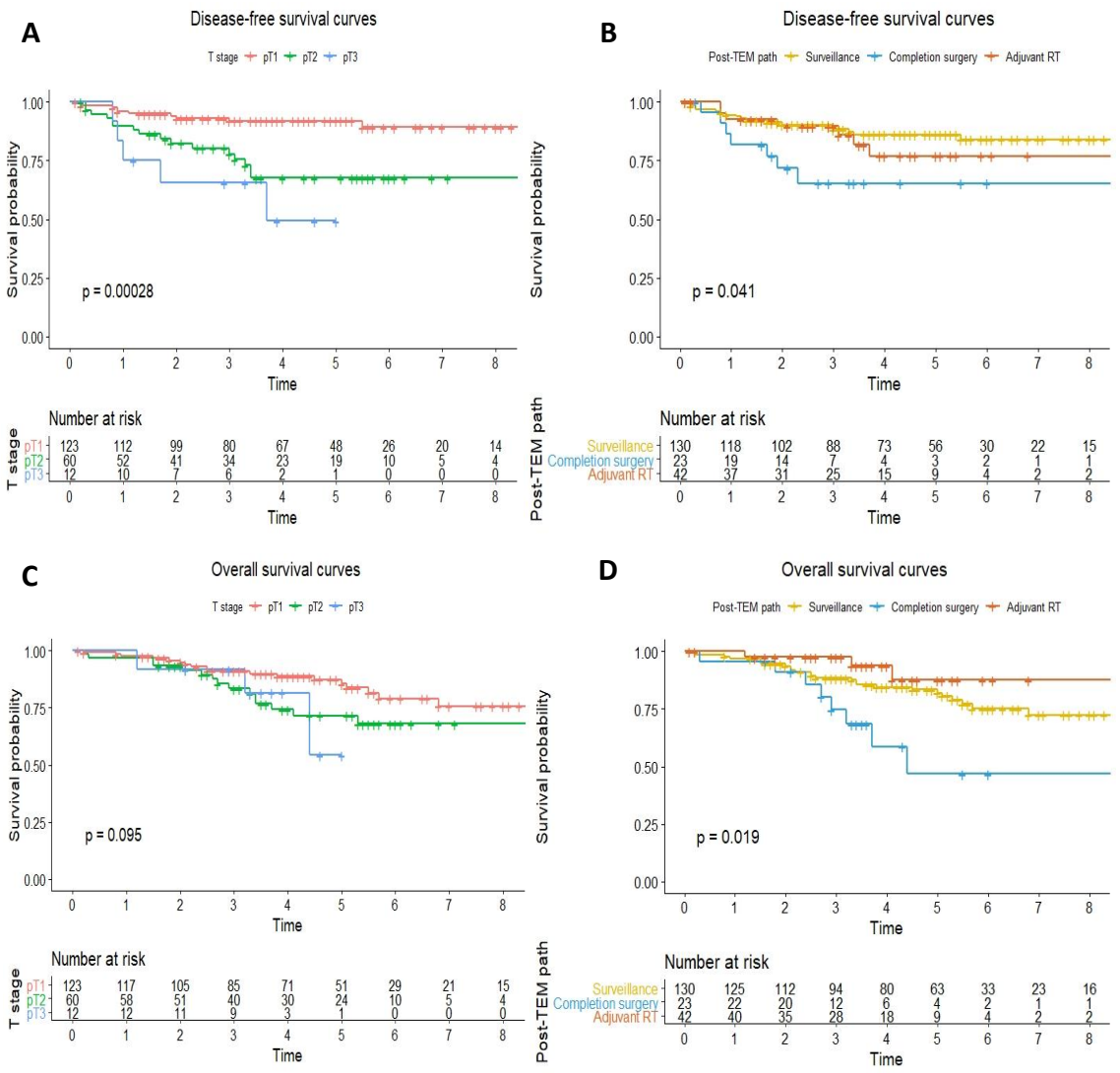


FIGURE 4.4: DISEASE-FREE AND OVERALL SURVIVAL CURVES AFTER TEM. A. DISEASE-FREE SURVIVAL BY TUMOUR STAGE. **B.** DISEASE-FREE SURVIVAL BY POST-TEM MANAGEMENT PATHWAY. **C.** OVERALL SURVIVAL BY TUMOUR STAGE. **D.** OVERALL SURVIVAL BY POST-TEM MANAGEMENT PATHWAY.

OVERALL SURVIVAL

The median follow-up time was 4.2 years for the 195 patients, excluding those who had pre-TEM radiotherapy (28) or no follow-up data (6). At last follow-up 13 patients (6.7%) had died of disease of disease at a median 3.3 years (range 2.1 – 5.3) following TEM and 23 patients (11.8%) had died from another cause, at a median of 2.1 years (range 0.2 – 9.6) following TEM. Twelve patients (6.2%) were alive with disease at a median 4.0 years (range 1.8 – 10.4) following TEM and 147 patients (75.4%) were alive with no evidence of disease at a median of 4.5 years (range 0.1 – 11.7)

following TEM. Kaplan-Meier estimates for overall survival are shown in Table 4.13, both by T stage of initial tumour and by post-TEM management pathway. Figure 4.4C & D show overall survival, again by T stage and by post-TEM management pathway.

TABLE 4.13: ESTIMATED OVERALL SURVIVAL AFTER TEM BY TUMOUR STAGE AND POST-TEM MANAGEMENT PATHWAY

	Number at risk	Estimated overall survival	Number at risk	Estimated overall survival	Number at risk	Estimated overall survival
	Tumour stage					
	pT1		**pT2**		**pT3**	
1 year	117	97.5% (95-100)	58	96.7% (92-100)	12	100% (100)
2 yrs	105	94.9% (91-99)	51	93.3% (87-100)	11	91.7% (77-100)
3 yrs	85	91.1% (86-97)	40	83.4% (74-94)	9	91.7% (77-100)
5 yrs	51	85.6% (79-93)	24	71.5% (60-86)	1	54.3% (23-100)
10 yrs	6	75.8% (65-88)	1	45.4% (20-100)		
	Post-TEM management pathway					
	Surveillance		**Completion surgery**		**Adjuvant radiotherapy**	
1 year	125	96.9% (94-100)	22	95.7% (88-100)	40	100% (100)
2 yrs	112	93.7% (90-98)	20	91.1% (80-100)	35	97.5% (93-100)
3 yrs	94	88.5% (83-94)	12	74.6% (58-97)	28	97.5% (93-100)
5 yrs	63	82.0% (75-90)	4	46.9% (25-87)	9	87.5% (74-100)
10 yrs	6	72.4% (63-84)			1	43.8% (11-100)

4.3.2 Prior neoadjuvant radiotherapy

Pre-operative radiotherapy was used in 28 patients. This group is analysed separately to those having primary TEM as they tend to have more advanced tumours; for seven (25%) TEM after radiotherapy was undertaken as a compromise rather than curative treatment. Furthermore, the tumour histopathological features may not represent the original tumour characteristics because of the radiation. Demographic, tumour and outcome details are shown in Table 4.14 and compared with the group that had primary TEM surgery. Significant differences were a higher incidence of late post-operative complications, a different distribution of excised tumour stage at TEM, and a three-times higher rate of local and distant recurrence in the neoadjuvant radiotherapy group.

TABLE 4.14: CLINICAL AND HISTOPATHOLOGY FEATURES OF PRE-TEM RADIOTHERAPY GROUP AND PRIMARY TEM GROUP

	Pre-TEM RT group	Primary TEM group	p value[+]
Number of patients	28	195	
Sex M:F	17(61%):11	121(62%):74	0.89
Age at TEM, median (range)	70 (43 – 86)	69 (31 – 94)	0.52
Operative time, minutes	83 (30-210)	75 (20-450)	0.65
Surgical defect sutured	16 (57%)	141 (72%)	0.10
Surgical complications			
Early	5 (18%)	27 (14%)	0.57
Late	9 (32%)	11 (6%)	0.000004*
Hospital stay, days	2 (1-19)	2 (0-71)	0.34
Tumour stage			0.018*[++]
ypT0	7 (25%)		
(y)pT1	5 (18%)	123 (63%)	
(y)pT2	14 (50%)	60 (31%)	
(y)pT3	2 (7%)	12 (6%)	
Distance from anal verge			0.11
≤6 cm	19 (68%)	101 (52%)	
>6 cm	9 (32%)	94 (48%)	
Tumour diameter (cm)			0.50
≤3.0	21 (75%)	156 (80%)	
3.1-5.0	5 (18%)	32 (16%)	
>5.0	2 (7%)	7 (4%)	
Differentiation			0.48
Well differentiated	1 (4%)	18 (9%)	
Moderately differentiated	24 (86%)	165 (85%)	
Poorly differentiated	3 (11%)	12 (6%)	
Lympho-vascular invasion	7 (25%)	70 (36%)	0.26
Resection margin positive	9 (32%)	68 (35%)	0.78
Local recurrence	9 (32%)	23 (12%)	0.0083*
Distant metastases	8 (29%)	18 (9%)	0.0074*
Follow-up (years), median (range)	3.9 (0.2-10.5)	4.2 (0.1-11.7)	0.69

[+]Chi-square or Fisher's exact test for categorical; t-test or Mann-Whitney for continuous,
[++] compares T0/1 v T2/3

Prior to commencing neoadjuvant radiation, the tumour was staged as cT1 in two, cT2 in 13, cT3 in 12 and cT4 in one; 12 (43%) had nodal metastases. CRT was used in 21 (75%) and short-course radiation in seven (25%). The median interval between completing radiotherapy and TEM was 17 weeks (range 9-59) for those who had CRT and 28 weeks (range 7-56) for those who had short-course RT. MRI assessment of tumour response six weeks after completing radiotherapy showed a very good initial response in 19 (68%), some response in seven and no response in two. Two of those with an initial complete response had TEM for recurrence.

In three cases, TEM surgery was noted to be more difficult than normal because of the prior radiotherapy; these operations took place 15, 16 and 39 weeks after completing radiotherapy. Late complications included five with TEM site dehiscence or non-healing with persisting sinus, three with stricture formation requiring repeated dilations and one who developed faecal incontinence and required a colostomy to relieve symptoms.

Of the seven with no residual tumour in the TEM specimen five had had CRT while two had short-course RT. Pre-operative staging was cT2 in three and cT3 in four.

The resection margin was positive in nine specimens – 32% overall, but 43% in patients with tumour present. Four of these patients were offered completion radical surgery; three refused and one was not done because distant metastases were detected prior to surgery. One patient had adjuvant post-TEM chemotherapy.

4.3.2.1 Outcome

One of the seven patients with no residual tumour at TEM developed local recurrence five months following TEM and subsequently also developed distant metastases. He underwent salvage APR but died of disease 1.8 years after TEM. The other six patients without residual tumour remain disease-free at a median of 6.6 years after TEM (range 3.0 – 9.4 years).

Of the 21 patients with residual or recurrent tumour present at TEM, 10 have developed tumour recurrence. These include three isolated local recurrence, two with distant metastases only and five with both local and distant disease. Recurrence was detected a median of 8.2 months (range 1.0 – 13.7) following TEM surgery. Two of the patients with isolated recurrence underwent salvage surgery, one low anterior resection and one Hartmann's procedure. One of these has no further evidence of disease at 9.9 years following TEM. Two of the ten patients were alive with disease at last follow-up (1.6 and 4.3 years after TEM), and seven had died of disease at a median of 3.5 years

after TEM (range 0.2 – 3.9 years). For the 11 with residual disease at TEM who have not developed recurrence, outcomes are good: nine are well at a median 4 years (range 2.3 – 10.5) after TEM and two died of unrelated causes at a median 7.7 years after TEM. The estimated local recurrence-free, disease-free and overall survival risks for the subgroup that had no residual tumour at TEM compared with those with disease present are shown in Table 4.15.

TABLE 4.15: ESTIMATED SURVIVAL AFTER TEM FOR PRE-TEM RADIATION GROUPS WITH OR WITHOUT RESIDUAL TUMOUR AT TEM

	No residual tumour, n=7		Cancer present in TEM specimen, n=21	
	Number at risk	Risk (95% CI)	Number at risk	Risk (95% CI)
Local recurrence-free survival				
Estimated risk				
At 1 year	6	85.7 (63-100)	15	70.0 (53-93)
At 2 years	6	85.7 (63-100)	12	65.0 (47-90)
At 3 years	6	85.7 (63-100)	10	65.0 (47-90)
At 5 years	5	85.7 (63-100)	5	55.7 (36-87)
Disease-free survival				
Estimated risk				
At 1 year	6	85.7 (63-100)	14	57.1 (40-83)
At 2 years	6	85.7 (63-100)	11	52.4 (35-79)
At 3 years	6	85.7 (63-100)	9	52.4 (35-79)
At 5 years	5	85.7 (63-100)	5	52.4 (35-79)
Overall survival				
Estimated risk				
At 1 year	7	100	20	90.5 (79-100)
At 2 years	6	85.7 (63-100)	18	90.5 (79-100)
At 3 years	6	85.7 (63-100)	14	73.5 (56-97)
At 5 years	5	85.7 (63-100)	6	60.1 (41-89)

Figure 4.6 shows survival curves for these groups and the primary TEM group. Outcomes for those with no residual tumour at TEM are equivalent to those for the primary TEM group and suggest there is not much value in undertaking TEM if there is no evidence of residual tumour on endoscopy or MRI scan following neoadjuvant radiotherapy. However, where there is residual tumour, outcomes are significantly worse, with almost half developing recurrent disease within a year of TEM, yet for those who survive the first year or so disease-free, outcomes are good.

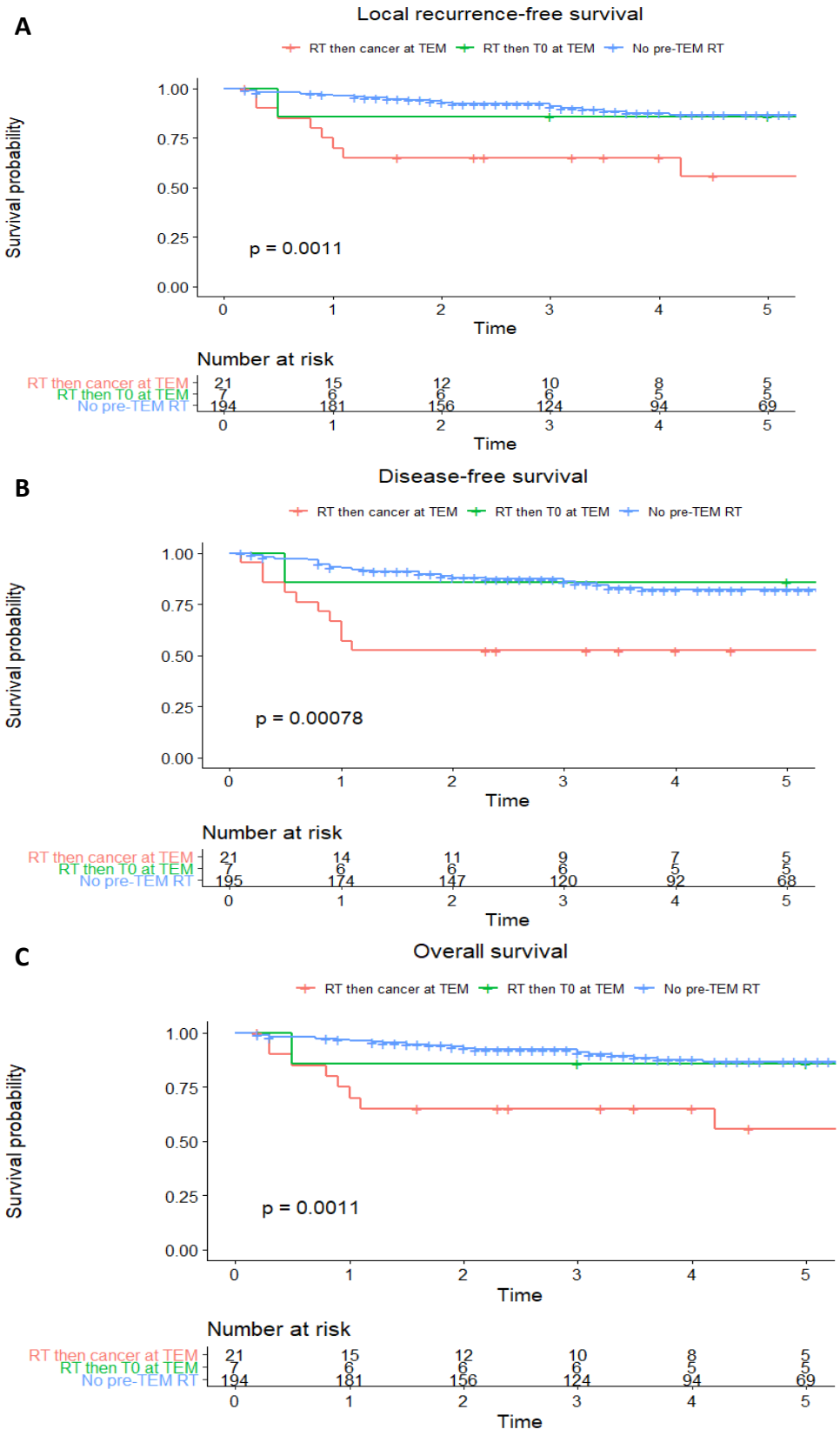


FIGURE 4.5: SURVIVAL CURVES FOR GROUP WITH PRE-TEM RADIATION WITH AND WITHOUT RESIDUAL TUMOUR AT TEM COMPARED WITH PRIMARY TEM GROUP. A. LOCAL RECURRENCE-FREE SURVIVAL. **B.** DISEASE-FREE SURVIVAL. **C.** OVERALL SURVIVAL.

4.3.3 Prior complete polypectomy

A previous polypectomy had been performed in 61 patients prior to TEM undertaken to remove the residual stalk or scar. No residual malignancy was found in 34 of these, and these are the focus of this subsection. The majority (21/34, 62%) had the polypectomy elsewhere and were referred for further management. Most (28/34, 82%) were undertaken prior to 2013, indicating a change in practice more recently. In five, TEM was undertaken because of residual or recurrent adenoma at the polypectomy site, while in the others the stated reason was to remove the scar or ensure clear margins as many polyps were fragmented – at least 11 (32%), or had involved polypectomy margins – at least 15 (44%). Almost half (16, 47%) had an endoscopy reported after polypectomy and prior to TEM, five of these showed residual adenoma while 11 were reported to show scar only.

Demographic, tumour and outcome details are shown in Table 4.16 and compared with the group that had primary TEM surgery to remove cancer. Significant differences were a shorter operative time, fewer early complications, smaller, better differentiated, earlier T stage tumours with less lymphovascular invasion, and longer follow-up. There was no difference in the incidence of positive resection margins, using the status of the original polypectomy, and although the rate of recurrence was lower this was not significant. Most polyps were sessile (20/29, 69%). Tubullovillous adenoma was the most common type (19/34, 56%). The median polyp size was 15mm (range 9 – 80). All were classed as pT1 on polypectomy histopathology.

In 14 of these TEM specimens without residual cancer, residual adenoma was present in the TEM specimen, whereas in 20 only scar tissue was found on histopathology. In the patients who had an endoscopy prior to TEM, three that were reported as showing scar only contained adenoma or dysplasia in the scar at TEM.

TABLE 4.16: CLINICAL AND HISTOPATHOLOGY FEATURES OF PRIOR COMPLETE POLYPECTOMY GROUP AND FOR TEM WITH CANCER GROUP.

	Prior complete polypectomy	Cancer present at TEM**	p value[+]
Number of patients	34	161	
Sex M:F	25(74%):9	96(60%):65	0.13
Age at TEM, median (range)	66 (31-83)	70 (38 – 94)	0.056
Operative time, minutes	60 (20-120)	88 (20-450)	0.00077*
Surgical complications			
Early	1 (3%)	27 (17%)	0.034*
Late	1 (3%)	10 (6%)	0.69
Hospital stay, days	2 (1-4)	2 (0-71)	0.12
Tumour stage			0.0000009*[++]
pT1	34 (100%)	89 (55%)	
pT1 sm1* **	6 (33%)	16 (10%)	
pT1 sm2* **	7 (39%)	24 (15%)	
pT1 sm3* **	5 (28%)	49 (30%)	
pT2	0	60 (37%)	
pT3	0	12 (7%)	
Distance from anal verge			0.32
<=6 cm	15 (44%)	86 (53%)	
>6 cm	19 (56%)	75 (47%)	
Invasive tumour diameter (cm)			0.024*
<=3.0	32 (94%)	124 (77%)	
>3.0	2 (6%)	37 (23%)	
Differentiation* **	[n= 20]		0.015*
Well differentiated	6 (30%)	12 (7%)	
Moderately differentiated	13 (65%)	138 (86%)	
Poorly differentiated	1 (5%)	11 (7%)	
Lympho-vascular invasion	5 (15%)	66 (41%)	0.0038*
Resection margin positive	15 (44%) [at polypectomy]	43 (27%)	0.043*
Local recurrence	2 (6%)	21 (13%)	0.38
Distant metastases	2 (6%)	16 (10%)	0.74
Follow-up (years), median (range)	5.3 (1.4-10.6)	4.0 (0.1-11.7)	0.0077*

**excludes patients who had pre-operative radiation, [+]Chi-square or Fisher's exact test for categorical, t-test or Mann-Whitney for continuous, [++]compares T1 v T2/T3, ***data not available for some cases

4.3.3.1 Outcome

Four patients (12%) developed recurrence. In two patients the recurrence was isolated local recurrence, one at 3 years and one at 9.5 years after TEM. Two patients developed distant disease only, one in liver and one widespread bone, liver and lung metastases. These developed at 1 and 10 years following TEM. In all four the original polyp type was tubullovillous adenoma, and three

contained high-grade dysplasia. The two local recurrences were from initial sessile polyps, one sm2 and one sm3, whereas the two distant recurrences were in pedunculated polyps, one Haggitt 3 and one Haggitt 4. One had a fragmented polypectomy and one had a positive margin at polypectomy. The patients who developed recurrence very late, 9.5 and 10 years after TEM, had no residual adenoma, only scar tissue in the TEM specimen. Those who developed recurrence within three years both had residual adenoma at TEM.

At a median follow-up of 5.2 years 25 patients were alive with no evidence of disease, five had died of other causes, two were alive with disease and two had died from recurrent rectal cancer.

Survival curves are shown in Figure 4.6 comparing three groups: those who had prior polypectomy with scar only at TEM, those with residual adenoma, and the main TEM group that did not have prior polypectomy. In general, the group with residual adenoma had similar outcome to the main group with cancer at TEM, whereas those with scar only appear to do better, however differences were not significant. On direct comparison between the prior polypectomy groups with scar only and residual adenoma, for disease-free survival the p value was approaching significance at 0.07, and for overall survival the p value was 0.02.

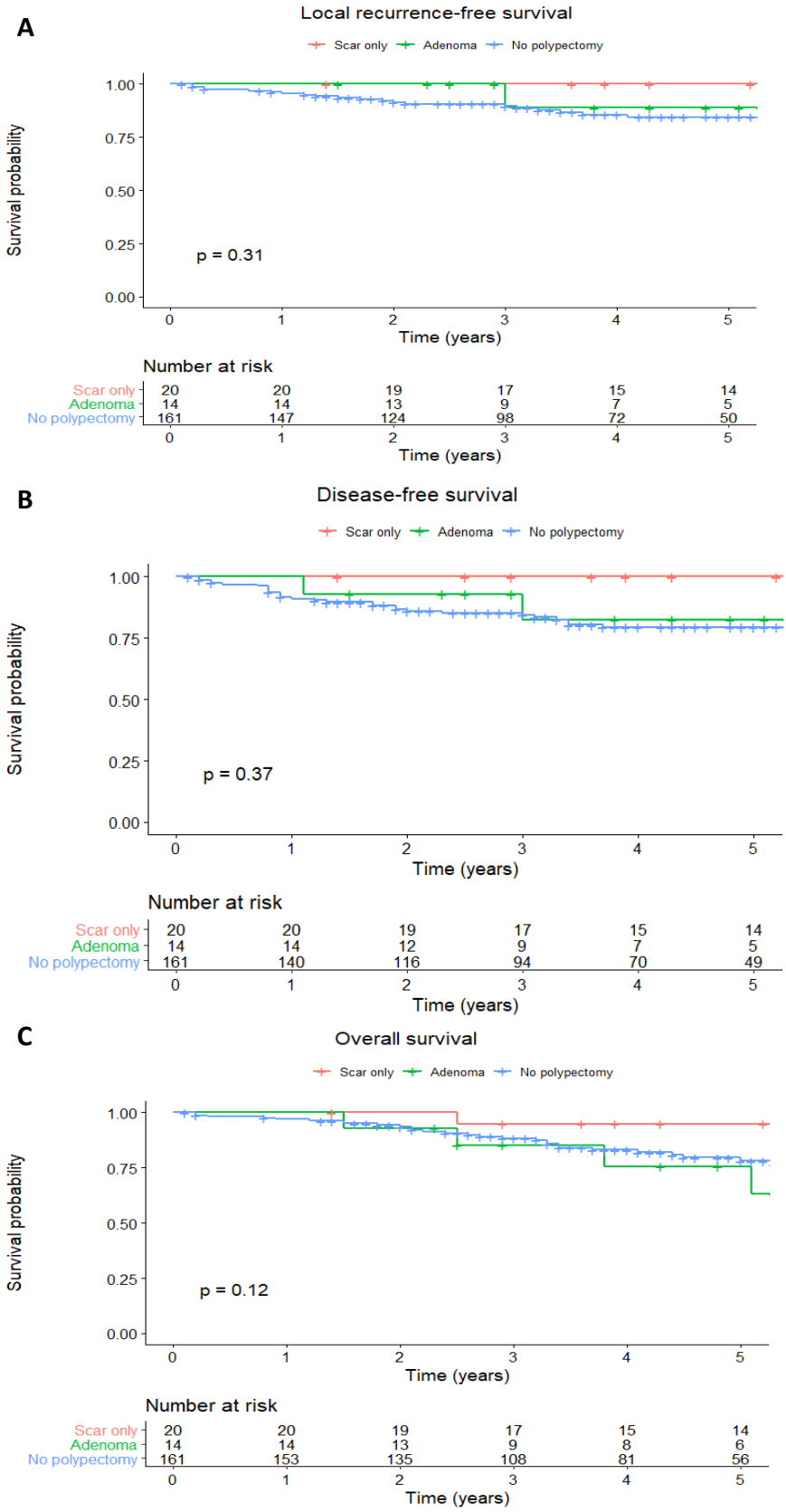


FIGURE 4.6: SURVIVAL CURVES FOR PRIOR POLYPECTOMY GROUPS WITH SCAR ONLY OR RESIDUAL ADENOMA COMPARED TO MAIN GROUP WITHOUT PRIOR POLYPECTOMY. A. LOCAL RECURRENCE FREE SURVIVAL. **B.** DISEASE-FREE SURVIVAL. **C.** OVERALL SURVIVAL.

4.3.4 Completion surgery

Early completion radical surgery was undertaken in 22 cases because of adverse histological factors. This group is considered separately as the absence of rectum and mesorectum affects the risk of local recurrence. Five of these patients were from out-of-region and had completion surgery done elsewhere; more limited data are available for this group.

A further two patients had radical surgery because of complications: one had a Hartmann's procedure four days after TEM because of perforation, and the other had low anterior resection with small bowel resection and ileal conduit because of complications of adjuvant radiotherapy. Neither developed disease recurrence; the first died of sepsis due to a parastomal abscess 2.9 years after TEM and the other was alive and well after 3.1 years.

Table 4.3 (Section 4.3.1.3) contains summary information for the completion surgery group. While there are no specific histopathological features that dictate post-TEM management, most of the tumours in the completion surgery group had higher T stage, and there was a relatively high incidence of lympho-vascular invasion, poor differentiation and resection margin positivity.

4.3.4.1 Completion procedure

For the 22 patients, completion surgery was carried out a median of 14 weeks following TEM (range 7 – 48 weeks). Three patients had radiotherapy after TEM and before the completion surgery, two because of pathology (T1sm3) in relatively young people and one because the patient initially refused completion surgery. The completion procedure was an anterior resection in 18 and APE in four. Of the anterior resections, four were planned open procedures, two were conversions, 11 were laparoscopic, three of which involved transanal total mesorectal excision (TaTME) and one was not specified. In six procedures, the operation was noted to be difficult, particularly the dissection around the previous TEM site, with bleeding noted in two and perforation of the TME specimen in three.

A comprehensive recording of complications was not available as some operations were undertaken elsewhere; the known complications are listed by Clavien-Dindo grade in Table 4.17. Five patients returned to theatre at least once because of post-operative complications; two had a Hartmann's for a leak, one had laparoscopy and revision of ileostomy and two had laparoscopic exploration with washout, one involving oversewing an anastomotic defect. One patient died of cardiac problems 33 days after surgery. The median length of hospital stay after completion surgery was 8 days (range 3 – 34).

TABLE 4.17: POST-OPERATIVE COMPLICATIONS AFTER COMPLETION SURGERY

Clavien-Dindo grade	Number	Complications after completion surgery*
I Any deviation from post-op course not requiring significant intervention	0	
II Requiring pharmacological intervention	2	1 AF 1 small bowel obstruction
IIIa Intervention not under GA	1	1 angioplasty after MI
IIIb Intervention under GA	5	2 Hartmann's for leak at 9 & 13 days 2 laparoscopic washout for leak at day 2 & 9 1 revision of ileostomy at day 6 1 multiple EUAs after leak
IVa Single organ failure	0	
IVb Multiorgan failure	0	
V Death	1	1 death at 33 days (had Hartmann's for leak)

*Some patients had multiple complications recorded

4.3.4.2 Pathology

For two patients who underwent completion surgery elsewhere full details of pathology are unavailable. In 11 completion procedures there was no residual tumour in the rectum or lymph nodes; the final staging in four of these was pT1sm3 and in seven was pT2. In two patients there was no residual tumour in the rectum, but tumour was found in the lymph nodes. The final staging for these was pT1sm3 N1. In seven patients there was residual tumour at completion surgery, this was pT2 in one, pT3 in five and pT4 in one. Four of these also had involved nodes. All resection margins were clear of tumour.

4.3.4.3 Outcome

Two patients (9%) developed local recurrence. Both also developed distant metastases and a further five patients developed distant metastases. All these seven patients had residual tumour in the completion specimen. The median time to first appearance of recurrence after completion surgery was 9 months (range 1 – 26). At last follow-up, five patients had died of disease at a median of 3.0 years (range 2.3 – 4.2) after completion surgery, two had died of other causes (at 0.1 and 1.7 years), two patient were alive with disease (at 3.1 and 5.4 years) and 13 patients were alive with no evidence of disease at a median of 2.7 years (range 1.2 – 5.2). Survival plots in Figure 4.3 and 4.4 compare survival for the three post-TEM management pathways. Comparing these groups, there was no significant difference in the incidence of local recurrence (p=0.14) but there were significantly more distant metastases (p=0.0002) in the completion surgery group.

4.3.5 Adjuvant radiotherapy group

Of the 223 patients undergoing TEM for rectal cancer where follow-up information is available, 42 (19%) had adjuvant radiotherapy because of adverse histopathological features; the majority of these were since 2013. Table 4.3 (Section 4.3.1.3) contains summary information for this group.

4.3.5.1 Radiotherapy

Radiotherapy commenced a median of 3 months following TEM, after complete healing of the TEM site had been confirmed endoscopically, and was completed 4.6 months (range 2.2 – 6.9) following TEM. In most patients, treatment involved CRT with 45 – 50.4 Gy delivered in 25 fractions with oral Capecitabine. Three patients had short-course radiotherapy (25 Gy in 5 fractions) without chemotherapy because of old age and frailty (two) or previous radiotherapy for an unrelated

condition (one). Some patients received treatment at another centre so full dosage details are not available.

Significant complications of radiotherapy were noted in two patients. One had persisting radiation proctitis 2.5 years after completing radiotherapy. The other developed small bowel infarction during radiotherapy, requiring surgery, and did not complete the full course. Comprehensive data on complications were not available, particularly for those receiving treatment at another centre.

4.3.5.2 Predicted local recurrence risk

PREDICTION ACCORDING TO PUBLISHED MODEL

The median predicted risk of local recurrence at three years for the 35 patients with well or moderately differentiated tumours and aged less than 80 years, according to a published model based on tumour stage, size and presence of lymphovascular invasion[14], was 21.4% (range 10.5% - 51%). Summing the risks for this group gives an expected incidence of local recurrence of eight events at 3 years. Among this group of 35 patients with a median 3.6 years follow-up, four instances of local recurrence were observed; this was significantly fewer (p=0.0002).

PROPENSITY SCORE MATCHING

Propensity scoring was used to identify a group of 42 patients from the 130 managed post-TEM by surveillance alone, that most closely matched the adjuvant radiotherapy group, with nearest match based on demographic and tumour characteristics. After inspecting the data, some variables were grouped: age and tumour height into three brackets and tumour size into four brackets for matching. Diagnostic plots are shown in Appendix 4.1. Table 4.18 compares the characteristics of the groups. In the matched surveillance group, there were ten instances of local recurrence (24%) at a median follow-up of 3.6 years compared with five (12%) in the radiotherapy group (p=0.008). The local recurrences were diagnosed at a median of 12 months post-TEM (range 3 – 41).

TABLE 4.18: CLINICAL AND HISTOPATHOLOGY FEATURES FOR PATIENTS HAVING ADJUVANT RADIOTHERAPY AFTER TEM AND FOR A PROPENSITY SCORE MATCHED GROUP HAVING SURVEILLANCE ALONE

PROPENSITY-MATCHED	Surveillance group	Radiotherapy group	p value[+]
Number of patients	42	42	
Sex M:F	30(71%):12	27(64%):15	0.48
Age at TEM, median (range)	69 (38 – 94)	68 (40 – 90)	0.78
Tumour stage			0.18
pT1	19 (45%)	14 (33%)	
pT2	21 (50%)	21 (50%)	
pT3	2 (5%)	7 (17%)	
Distance from anal verge			0.13
≤6 cm	19 (45%)	26 (62%)	
>6 cm	23 (55%)	16 (38%)	
Tumour diameter (cm)			0.45
≤3.0	33 (79%)	28 (67%)	
3.1-5.0	7 (17%)	12 (29%)	
>5.0	2 (5%)	2 (5%)	
Differentiation			1
Well differentiated	1 (2%)	2 (5%)	
Moderately differentiated	38 (90%)	38 (91%)	
Poorly differentiated	3 (7%)	2 (5%)	
Lympho-vascular invasion	19 (45%)	26 (62%)	0.13
Resection margin positive	14 (33%)	18 (43%)	0.37
Local recurrence	10 (24%)	5 (12%)	0.0083*
Distant metastases	5 (12%)	5 (12%)	1
Follow-up (years), median (range)	3.6 (0.2-8.3)	3.6 (0.1-10.1)	0.85

[+] Chi-square or Fisher's exact test for categorical, Mann-Whitney for continuous

4.3.5.3 Outcome

Median follow-up was 3.6 years (range 0.1 – 10.1) for the radiotherapy group. There were five instances of local recurrence (12%), two in pT1sm3 stage tumours, two in pT2 and one in a pT3 tumour. These occurred at a median 23 months (range 6 – 45) following TEM, almost double the interval in the matched group, although not reaching significance. Two local recurrences were isolated, one developed local recurrence seven months after distant disease, and two developed distant disease after local recurrence. Four were being followed up elsewhere; in some cases, review of earlier scans, prior to detection of recurrence, suggested an opportunity for earlier detection had been missed. This reinforces the need for meticulous surveillance.

The isolated recurrences were both treated by salvage surgery, one pelvic exenteration and one APE. At most recent follow-up both are disease-free. Of the three who also had distant disease, one had liver resection. Kaplan-Meier estimates of local recurrence in this group were 0% at one year, 5.5% at 3 years and 20% at 5 years (Table 4.4).

In total five patients in the radiotherapy group developed distant disease, two without local recurrence. The distant disease was diagnosed a median of 10 months (range 9 – 45) following TEM; two involved the liver only, one lung only and two had widespread disease.

Overall for the radiotherapy group at a median 3.6 years follow-up, 35 (83%) were alive with no evidence of disease at last follow-up, three (7%) were alive with disease, two (5%) had died of disease (3.3 and 4.1 years post-TEM) and two (5%) had died of other causes.

COMPARISON WITH PROPENSITY SCORE MATCHED GROUP

Survival estimates for the radiotherapy and matched surveillance groups are shown in Table 4.19.

TABLE 4.19: ESTIMATED SURVIVAL AFTER TEM FOR THOSE WHO HAD ADJUVANT RADIOTHERAPY AFTER TEM AND A PROPENSITY SCORE MATCHED GROUP FOLLOWED BY SURVEILLANCE ALONE

PROPENSITY-MATCHED	Surveillance group		Radiotherapy group	
	Number at risk	Risk (95% CI)	Number at risk	Risk (95% CI)
Local recurrence-free survival				
Estimated risk				
At 1 year	34	87.7 (78.2-98.4)	40	100
At 2 years	24	79.7 (68.0-93.4)	33	94.5 (87.4-100)
At 3 years	19	79.7 (68.0-93.4)	26	94.5 (87.4-100)
At 5 years	10	70.8 (56.3-100)	9	79.6 (64.2-98.6)
Disease-free survival				
Estimated risk				
At 1 year	33	85.3 (75.0-96.9)	37	92.5 (84.7-100)
At 2 years	22	74.6 (62.2-89.6)	31	89.6 (80.4-99.8)
At 3 years	19	74.6 (62.2-89.6)	25	89.6 (80.4-99.8)
At 5 years	10	66.3 (51.8-84.8)	9	77.0 (62.7-94.5)
Overall survival				
Estimated risk				
At 1 year	39	95.2 (89.0-100)	40	100
At 2 years	32	90.4 (81.8-99.8)	35	97.5 (92.8-100)
At 3 years	24	75.7 (62.9-91.0)	28	97.5 (92.8-100)
At 5 years	15	69.2 (55.4-86.5)	9	87.5 (74.3-100)

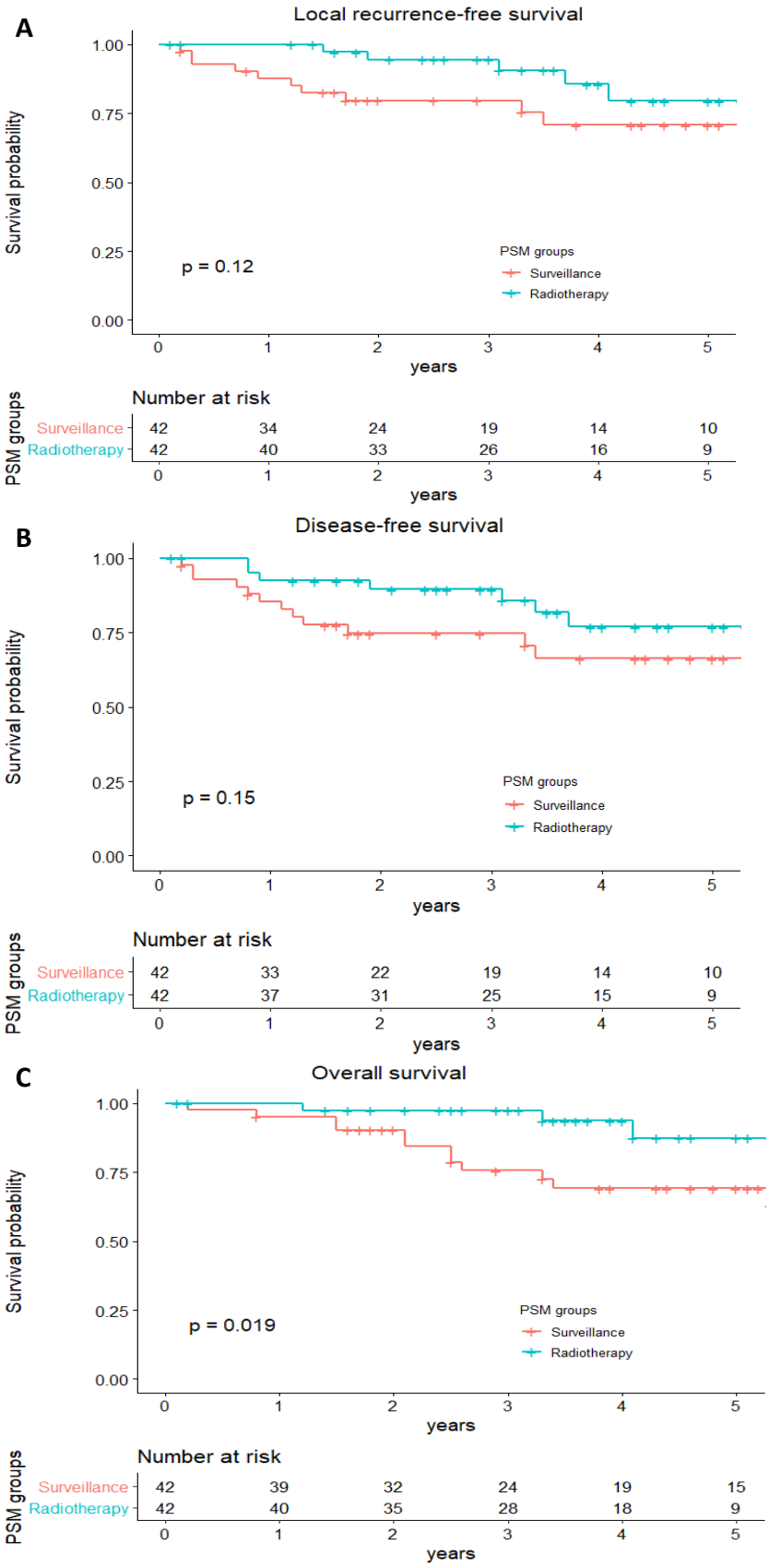


FIGURE 4.7: SURVIVAL CURVES FOR GROUP HAVING ADJUVANT RADIOTHERAPY AFTER TEM COMPARED WITH A PROPENSITY SCORE MATCHED GROUP FOLLOWED BY SURVEILLANCE ALONE. A. LOCAL RECURRENCE FREE SURVIVAL. **B.** DISEASE FREE SURVIVAL. **C.** OVERALL SURVIVAL.

Survival curves comparing the adjuvant radiotherapy and propensity-matched surveillance only groups are shown in Figure 4.7. Differences in local recurrence-free and disease-free survival are not significant, although the figures do suggest a delay till disease recurrence after radiotherapy. For overall survival, the radiotherapy group have a significantly better outcome (p=0.02).

4.3.6 National perspective on local excision for early rectal cancer

The survey was emailed to 228 colorectal MDT contacts around the UK; 28 email addresses were no longer valid. From the 200 valid emails, 45 questionnaires (23%) were returned. While this cannot be considered representative of colorectal MDTs in the UK generally, responses to a survey on early rectal cancer (ERC) will be skewed towards units that manage ERC so it may be more representative of those units.

Of the 45, 21 (47%) stated that their MDT managed all cases of ERC, 15 (33%) said their MDT managed most but referred selected cases elsewhere, and 9 (20%) referred most or all cases of early rectal cancer elsewhere.

The majority of units (36, 80%) stated they offered local excision for ERC although three provided additional comments indicating reservations with this strategy. One stated ‘we do not recommend local excision of early rectal cancer in fit patients’ but would do it if patient wanted it and understood that it was not ‘gold standard treatment’. Another only offers local excision to unfit patients with ERC and would refer patients who are fit and do not want radical surgery elsewhere. The third only considers local excision acceptable for very early cancer – ‘T1sm1 with no adverse features’ – and since these cannot be identified in advance sees local excision as ‘poor practice’.

Figure 4.8A indicates the local excision techniques offered by these units to manage ERC, showing that over half (26, 62%) offered endoscopic removal. Of the three modern transanal endoscopic surgical techniques, TEMS was slightly more common than TAMIS, with TEO available less

commonly. Traditional transanal excision was offered by over a third (16, 38%). Two units commented on the difficulties of managing patients referred after incomplete or inappropriate endoscopic removal.

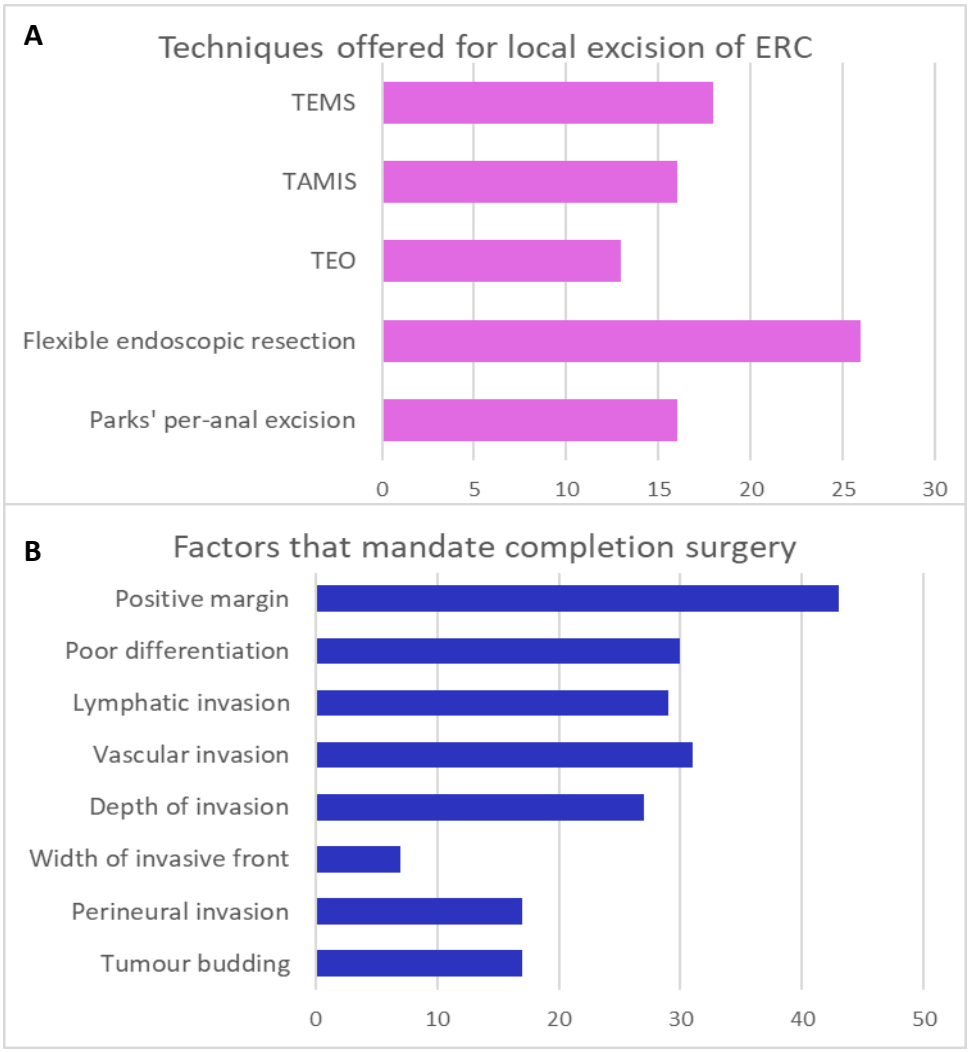


FIGURE 4.8: RESPONSES TO NATIONAL MDT SURVEY ON MANAGEMENT OF EARLY RECTAL CANCER. A. TREATMENT OPTIONS OFFERED. **B.** HISTOPATHOLOGY FEATURES CONSIDERED TO BE AN ABSOLUTE INDICATOR FOR COMPLETION SURGERY.

After a patient has had local excision, all but one unit were able to provide surveillance, and all but two provided completion surgery. Most (38, 86%) were able to provide CRT although one stated that the local oncologist did not support this strategy, so patients were referred elsewhere if this was considered appropriate. A third (16, 36%) offered contact radiotherapy and two stated they referred patients elsewhere if this was indicated.

Respondents were asked whether they had rigid criteria for proceeding to completion surgery after local excision in patients who were fit for major surgery. Figure 4.8B shows the responses. All but one (96%) considered a positive resection margin as an absolute indicator. For those who considered depth of invasion the most common cut-off was sm3 (31/35, 89%) while the remainder used sm2. Several commented that no single factor mandated treatment, but all factors were taken into account in the discussion at MDT and with the patient. One stated that they would not undertake completion surgery unless there was obvious residual or recurrent disease, regardless of any high-risk features. Comments indicated that some MDTs still rely on the local recurrence risk model published over a decade ago[14].

For patients who had these high-risk features and were fit but did not want completion surgery 38 (84%) would offer CRT followed by surveillance and 25 (56%) would offer surveillance alone. A quarter (11, 24%) would offer referral to another centre for further management. One unit commented that CRT was offered despite there being no evidence to support it at present because 'offering something seems the right thing to do'.

For patients who were not fit for completion surgery, more would favour CRT (40, 89%) in preference to surveillance alone (24, 53%). The same proportion would offer referral to another centre (11, 24%).

Finally, MDTs were asked if they would consider taking part in a clinical trial randomising patients after local excision of ERC between surveillance alone or CRT then surveillance. The vast majority (40, 89%) said yes, but some expressed reservations, one saying they would only be comfortable randomising patients with T1sm2 stage tumours, and two saying participation would depend on the details of the trial. Several MDTs commented that they already participate in the STAR-TREC trial[20] which randomises patients with early rectal cancer, up to T3, between the more invasive options of radical surgery or neoadjuvant radiation.

4.4 Discussion

This study covers 229 TEM procedures for cancer in a single high-volume institution over an 11-year period. Local recurrence rates are lower than expected, and trends over time show fewer TEMs after complete polypectomy and more adjuvant radiotherapy in the latter half of the period.

4.4.1 Local recurrence after TEM

The overall local recurrence rate in this series was 12% (23/194) with estimated 3-year local recurrence rate of 6% for T1 and 16% for T2 tumours. These rates are considerably less than an older published series with rates of 13% and 27% respectively[14], yet which the national survey suggests is still relied on by some MDTs in advising patients about their risk of local recurrence and deciding on a post-TEM management pathway. This older model was based on a series of 424 procedures done at 21 centres between 1993 to 2006, in the early days of modern local excision, so it is only to be expected that outcomes will improve over time as experience grows and is consolidated. Improvements in pre-operative imaging which now allow for more rigorous patient selection undoubtedly also contribute to the improvement. Given this, it is surprising that the survey shows a persisting view among some MDTs that local excision alone is unsafe except in very early disease. This demonstrates a lack of confidence in adjuvant treatment or surveillance to minimise the effects of residual microscopic disease by preventing local recurrence or detecting it, in the small number of cases when it does occur, sufficiently early to avoid an adverse outcome for the patient. The present results for salvage surgery do not completely support this pessimism. Yet it must be acknowledged that patients who do experience local recurrence have a worse outcome than those who have primary radical surgery. One third of these (8/23) also developed distant disease and while some tumours are intrinsically aggressive, leaving undiagnosed malignancy in the mesorectum for longer no doubt contributes to this high incidence of distant disease. The more extensive surgery, with radio- and chemotherapy, that is required to treat those who develop

recurrence must be discussed with the patient prior to embarking on an organ-preserving treatment pathway for early rectal cancer, as the occurrence of local recurrence will significantly disadvantage the patient compared to undergoing primary radical surgery.

SALVAGE SURGERY

In the present series salvage surgery was undertaken in 14 of the 23 patients who developed local recurrence after TEM. Four required some form of exenteration, and for the ten with available data, six had a T3/4 stage tumour and four had positive resection margins. These figures and anecdotal observations indicate that the recurrent tumour was not detected as early as it might have been, reinforcing the need for a rigorous surveillance regime, particularly where patients are followed up at a centre with less experience in managing these patients. Nevertheless, the available information indicates a lower incidence of post-operative complications after salvage surgery than after completion surgery, probably because the procedures were taken considerably later after the local excision, a median of 2.1 years, compared with 3.2 months for completion surgery, allowing time for the fibrosis induced by the TEM procedure to resolve. Half of the patients (7/14) who underwent salvage were alive with no further sign of recurrence at a median of 5.2 years after the initial TEM, and a median 4.0 years following the salvage procedure. Three of these had a permanent stoma and the other four had restored continuity after closure of an ileostomy.

The estimated overall survival rates after salvage surgery in this series are lower than after primary radical surgery: 62% at 5 years compared with 81% found by a recent analysis for T1/T2 tumours in the US National Cancer Database[156]. However, if survival is measured from initial TEM procedure, the 5-year 84% rate in the present series is much closer.

As the median age for TEM excision is 69 years, and the procedure is associated with fewer complications and less impact on quality of life than radical surgery, an 84% 5-year survival rate after TEM despite the occurrence of local recurrence then a radical salvage procedure may not be

considered unfavourably by some patients or clinicians. Indeed, in view of the relatively poorer outcomes after completion surgery, meticulous surveillance after TEM with salvage surgery as soon as early recurrence is identified may in some cases be preferable to potential over-treatment with completion surgery, even in the presence of high-risk histopathological features. Certainly, it is essential to fully discuss the potential outcomes with the patient following the diagnosis of early rectal cancer, particularly if the patient is averse to major surgery or has comorbidities. And it must always be borne in mind that recurrence after TEM will have a significant negative impact on the patient.

4.4.1.1 Risk factors for local recurrence

In keeping with previous reports[14,16], tumour stage, size and lymphovascular invasion were significant factors for local recurrence. Interestingly, a positive resection margin did not feature in the univariate analysis, nor did the post-TEM management pathway. This may be because many patients with a positive resection margin (26/43, 60%) underwent either adjuvant radiotherapy or completion surgery, which is expected to mitigate the effects of a positive margin at TEM.

POSITIVE MARGINS

There was a relatively high incidence of positive resection margins (24%) in this series, although in at least 28% of these the positivity was based on tumour present within 1mm of the margin rather than directly involving the margin. This rate is higher than some recently published series[157-159], although direct comparison is difficult due to different inclusion criteria, different definitions of positivity, and variable length of follow-up. The high margin-positive rate in the present series is disappointing and reinforces the need for improved pre-operative staging, since the margin positivity increases markedly with higher tumour stage. However, the intention to achieve full-thickness excision and so avoid deep margin involvement may at times not be achievable due to

the proximity to the sphincter muscle in low rectal cancers or the need to avoid peritoneal breach in higher cancers. With adequate staging, patients can be advised of this risk prior to surgery and make an informed decision whether to pursue an organ-preserving approach with a higher recurrence risk and potential need for further subsequent treatment or to undergo primary radical surgery.

NEURAL INVASION

Neural invasion featured highly in factors associated with recurrence, being more significant and with greater effect size (hazard ratio = 5) than any factor other than T stage. This is not often addressed in studies of rectal cancer but has been shown to be a significant prognostic factor and is on the RCP core data items (Table 1.1). Liebig *et al.*[160] initially found that it was identified in less than 0.5% of pathology reports, but on review they found 22% of colorectal tumours, and 30% of rectal tumours, exhibited perienural invasion. While it correlated with other adverse factors such as tumour stage and grade of differentiation, it was an independent prognostic factor for disease-free and overall survival. They also noted that half of patients with perineural invasion had metastases at the time of diagnosis. While not all studies have confirmed a relationship, a systematic review[161] covering 58 articles found that perineural invasion was present in 21% of rectal tumours and was an independent prognostic factor in disease-free and overall survival. A metanalysis covering 19 studies and over 6,000 patients[162] found perineural invasion was a negative prognostic factor for overall survival (HR 1.30) and disease-free survival (HR 2.14). Kim *et al.*[163] found that neoadjuvant CRT did not affect the incidence of detection of perineural invasion, in contrast to lymphovascular invasion which was reduced from 21% to 8% after CRT, so they conclude that perineural invasion remains a useful prognostic factor in patients treated with neoadjuvant CRT. Pagès *et al.*[164] noted that perineural invasion is an early sign of invasiveness, so its prominence in the present series may be explained as these locally excised tumours are generally early rectal

cancers. These findings suggest that it is worth ensuring neural invasion is systematically sought on histopathological analysis and given due consideration in planning a patient's post-TEM management pathway in this group with early rectal cancer.

4.4.2 Post-TEM management pathways

In the present series two-thirds of patients embarked on a surveillance regime after TEM while 11% had planned completion surgery and 22% had adjuvant (chemo-)radiotherapy. The incidence of local recurrence was the same in all treatment groups (Figure 4.3B) while distant metastases were more common in the completion surgery group and less common after adjuvant radiotherapy (Table 4.3), reflected in significantly different disease-free and overall survival (Figure 4.4B&D). However, the number of patients having completion surgery was fairly low which limits confidence in the conclusions.

4.4.2.1 Completion surgery

Completion surgery is currently the standard post-TEM treatment in the presence of adverse histopathological features[17], and the comments in the MDT survey indicate it is strongly favoured in the presence of positive resection margins, poor differentiation and vascular invasion. Completion surgery in the current series was associated with a relatively poor outcome. Post-operative complications were common, consistent with the increased surgical difficulty of a radical procedure soon after local excision. Other studies have also noted high operative morbidity[136,165]. The newer procedure of transanal TME (TaTME) may allow completion surgery with less morbidity. Koedam *et al.*[166] compared short-term outcomes for 25 patients having completion by TaTME with a matched group having standard TME. They noted less perforation, a higher lymph node yield, less short-term morbidity and shorter hospital stay after TaTME.

Van Groningen *et al.*[167] reviewed all local excisions recorded in the Dutch Colorectal Audit, a compulsory national database of colorectal cancer procedures 2012-2015. Over four years, 448 primary local excisions were done; the guidelines stipulated that 239 of these should have had completion TME surgery because the tumour was high-risk pT1 or pT≥2. In fact, only 68 (28%) had completion surgery: 13% for high-risk T1, 31% for pT2 and 70% for pT3/4 tumours. Only six of the remaining patients had adjuvant radiation. Their paper does not explain the reason for the low level of compliance with the guideline, but the poor outcomes demonstrated in the present series, with high morbidity and significantly worse disease-free and overall survival following completion surgery compared with adjuvant radiotherapy or surveillance alone, may justify a reluctance to advocate completion surgery. The ongoing TESAR trial[21] will provide evidence to better inform decisions about this pathway.

4.4.2.2 Adjuvant radiotherapy

Adjuvant radiotherapy was used in almost one quarter of this series and has become increasingly common in recent years. The MDT survey confirmed that this practice is widely accepted in the UK despite a lack of rigorous evidence for its benefit. While completion surgery is the 'gold standard' pathway patients may not be fit for radical surgery, or may prefer an organ-preserving option, especially when the risks of completion surgery are known. One aim of this chapter is to provide some evidence regarding this pathway.

The rate of local recurrence in this series was not significantly different from the other two pathways looking at the raw numbers. However, when compared with a propensity score matched group to adjust for the presence of risk factors, the incidence of local recurrence was halved compared to surveillance alone. While the propensity score matching is not perfect, as the radiotherapy group are selected for adjuvant treatment based on histopathology findings, demographic and tumour characteristics were not significantly different between the groups.

Another interesting feature is that local recurrence occurred later in the radiotherapy group (median 23 months) compared to the surveillance alone group (median 12 months) although this did not reach significance. This raises the possibility that radiotherapy may delay, rather than just prevent, local recurrence in these high-risk patients, so a longer duration of follow-up would be required. Yet, in an elderly, comorbid population, simply extending the duration of local control by the use of adjuvant radiotherapy after local excision may have practical advantages compared to radical surgery.

The rationale for post-TEM adjuvant radiotherapy is to treat a risk of residual cancer within the rectum and mesorectum that is not identified on standard staging, and therefore must be low volume. The availability of definitive histopathological staging based on the TEM specimen means that inappropriate use of radiotherapy for early stage disease is avoided. The disadvantage of radiotherapy in this setting compared with neoadjuvant treatment is that there is no visible tumour to be able to measure response, and, as in any setting, it cannot be predicted whether the cancer cells are sensitive to radiation, so patients may receive radiotherapy, and its potential risks, without benefit.

4.4.3 TEM after other treatments

In almost one-fifth (41/223, 18%) of this series, there was no residual tumour at TEM following either polypectomy or neoadjuvant radiation. The question arises as to whether the TEM procedure is necessary in this situation.

4.4.3.1 Neoadjuvant radiation

After neoadjuvant radiation, if there is residual tumour the outcome is significantly worse than for patients having a primary TEM, as might be expected from the more advanced disease which

directed the need for neoadjuvant treatment in the first place. These patients have a high rate of recurrence (10/21), and this is generally within the first 12 months after TEM. A contributory factor to this high recurrence rate may be positive resection margins, although the observed incidence was the same as in primary TEM cases. It is recognised that following radiation the residual tumour may be fragmented within the bowel wall. Perez *et al.*[168] observed that occult lateral spread of tumour beneath normal mucosa is a common finding after CRT. They recommend taking a margin of 1.5cm around the visible tumour to reduce the positive margin rate but note that there will still be instances of residual tumour beyond this distance. So even when margins are reported clear, microscopic tumour may remain.

Local excision after radiation is a more difficult procedure than primary surgery and is associated with a high rate of late complications. These are all factors worth bearing in mind when discussing with patients with residual tumour how to proceed, although in the presence of comorbidity options may be limited. And it is still worth noting that half do not develop recurrence.

The picture is different where there is no residual tumour in the TEM specimen. Recurrence rates are very low, although the numbers here are too low for a definitive conclusion. Even so, it would seem prudent to fully advise patients of the potential surgical risks before deciding to undertake local excision of the residual scar when there is no evidence of residual tumour on MRI scan or endoscopy.

The role of pre-operative radiotherapy still needs clarification and ongoing trials are addressing this, for example STAR-TREC[20] is randomising patients with early rectal cancer between radical surgery and an organ-preserving strategy with either long-course CRT, or short-course radiotherapy followed by surveillance, local excision or radical surgery based on response to neoadjuvant therapy.

4.4.3.2 Complete polypectomy

Endoscopic removal of a polyp cancer may yield a fragmented specimen with positive or uncertain resection margins. It has been suggested that after polypectomy with unsatisfactory margins, local excision should be done as it is a minor procedure and removes the risk of recurrence[169,170]. It is debatable whether TEM or other means of local excision constitutes a minor procedure, and there is a recognised morbidity. The present results also show that the risk of recurrence, though low, persists; of 34 patients with no residual cancer four developed recurrence, and in two this was around 10 years later.

The earlier recurrences both happened in patients where, although there was no residual cancer in the TEM specimen, there was residual adenoma. Numbers in these subgroups were too small for rigorous analysis but it appears that those with residual adenoma at TEM have similar outcomes to those having primary TEM for cancer whereas in the absence of residual adenoma, where there is scar only in the TEM specimen, the recurrence rate is so low that the TEM procedure does not confer any benefit. One potential problem with deciding to undertake TEM or not according to presence of residual disease is the accuracy of the post-polypectomy endoscopy: in three cases that were recorded as scar only on endoscopy, adenoma or dysplasia was present in the TEM specimen. These cases were in the early years of the study, prior to 2012, and it is possible that newer endoscopic techniques would more accurately diagnose the presence of residual disease.

The very late recurrences are a cause for concern. The original polyp cancers were small and early, and completely removed according to histopathology. This demonstrates that there is a small subgroup of cancers that are particularly aggressive, seeding early, before the polypectomy, and many years later developing extensive recurrence. It is not feasible to subject all patients with small polyp cancers to decades of follow-up with scans and endoscopy when the incidence is so low, yet the possibility of very late recurrence needs to be borne in mind. The ideal way to address this issue would be to identify a marker for aggressive disease in the original polyp and follow closely only

those with aggressive potential. Such a marker does not yet exist, but will no doubt be found eventually, and ideally would be easily assessed, for example a blood test for a molecular marker.

4.4.4 Future directions

The precise role for adjuvant radiotherapy following local excision requires further clarification through larger-scale assessments of long-term outcome and clinical trials. The ongoing TESAR trial[21], comparing outcomes for patients having local excision of early rectal cancer followed by either completion surgery or adjuvant CRT because of adverse pathological features will provide some clearer evidence of the place for radiotherapy following local excision. The national MDT survey indicates a strong interest in a trial randomising patients after local excision of early rectal cancer between surveillance alone, versus adjuvant (chemo)- radiotherapy and surveillance. As a follow-up to the present study, the investigators are in the process of setting up a clinical trial, Vorates, to this effect. In this trial it will be important to ensure sufficient length of follow-up due to the evidence here that radiotherapy may delay the development of recurrence. There is also a need for a marker of radiosensitivity or radioresistance so the locally excised cancer can be examined, and adjuvant radiotherapy only considered in those who have the potential to benefit.

An important issue not yet addressed is the patients' quality of life after TEM and some of the treatment pathway variations mentioned in this chapter. This is addressed in Chapter 7.

The next chapter will analyse the molecular data available for many of the patients covered in the TEM database and look further for prognostic factors that can help in deciding how to manage early rectal cancer.

Chapter 5: Digital and molecular prognostic factors

5.1 Introduction

This chapter describes a number of experiments using tissue samples from patients in the TEM database described in the previous chapter to investigate digital and molecular features and correlate these with clinical outcome.

The main objective of this chapter is to identify prognostic factors, using digital or molecular methods, on tissue from locally excised rectal cancers, that can identify patients at high risk for recurrence after local excision. This would allow these patients to be specifically targeted for adjuvant treatment or closer surveillance.

Background for this chapter is contained in Section 1.3.

5.1.1 Aims

1) To identify digital markers of poor prognosis in TEM tissue samples

2) To assess whether markers of a TSA precursor polyp provided prognostic information

3) To identify molecular markers of poor prognosis in TEM tissue samples

4) To identify molecular markers of resistance to adjuvant radiotherapy in TEM tissue samples

5.2 Methods

All methodology is described in Chapter 2: Materials and methods.

5.3 Results

Suitable TEM tissue samples were available from 169 of the patients in the database presented in the previous chapter. For 12 patients there was also at least one additional subsequent sample;

these included 10 total mesorectal excisions with residual or recurrent disease and five excisions of metastases, one each of lung, spleen, and perineal skin and two liver resections. A further three additional samples were available in cases where the original TEM sample was not available for molecular data.

5.3.1 Potential markers of poor prognosis

5.3.1.1 Extra histopathology data

MSI STATUS

MSI status was available for 56 TEM samples. Only one (2%) showed loss of any MMR (PSH2). Another was known to have MSH2 positive HNPCC and ulcerative colitis. Both had good outcomes. The rarity of loss of expression of any of the four DNA mismatch repair genes in this rectal cancer series imply it is not a valuable prognostic feature in this setting and is consistent with the expected 2% incidence of MSI in rectal cancers[33].

GENE PANEL MUTATIONS

KRAS: Results were available for 58 samples. In 26 (45%) a mutation was detected. In most this was an activating mutation in codon 12/13; in the remainder a mutation elsewhere, of uncertain significance, was detected.

BRAF: Results were available for 58 samples. A mutation, of uncertain significance, was detected in two (3%). Again, this low level is expected in rectal cancer[41].

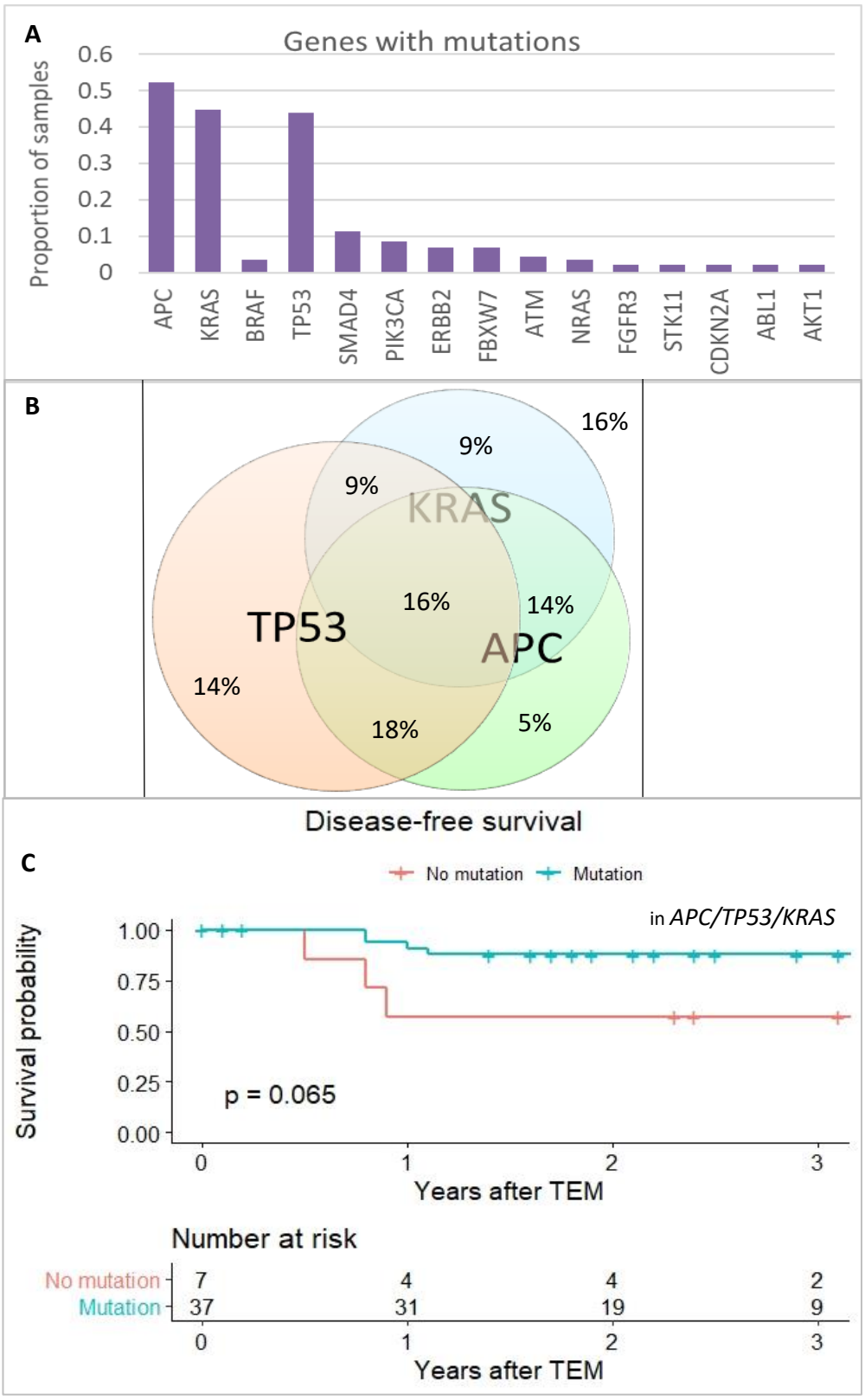


FIGURE 5.1: GENES FROM THE 50-GENE PANEL CONTAINING MUTATIONS IN 44 TEM SAMPLES. A. BAR CHART SHOWING ALL GENES OF THE 50 THAT CONTAINED A MUTATION. **B.** VENN DIAGRAM SHOWING PROMINENCE OF THE THREE MOST COMMON GENE MUTATIONS, AND OVERLAP. ONLY 16% OF THE SAMPLES DID NOT CONTAIN A MUTATION IN AT LEAST ONE OF THE THREE GENES. **C.** DISEASE-FREE SURVIVAL ACCORDING TO WHETHER THERE WAS A MUTATION PRESENT IN AT LEAST ONE OF *APC/TP53/KRAS* OR NO MUTATION IN THESE THREE GENES.

The more extensive 50-gene panel was used to test 44 samples. No additional mutations were detected in 14. The most common genes that showed mutations were *APC* (51%) and *TP53* (44%); 75% of tumours showed an *APC* and/or *TP53* mutation. These are tumour suppressor genes known to be important in colorectal cancer. Loss of *APC* function is an early step in the adenoma-carcinoma sequence, causing activation of the Wnt pathway while *TP53* acts to regulate gene expression and prevent mutation.

All genes that showed a mutation are shown in Figure 5.1A.

No gene mutation individually had a significant association with disease recurrence. Of the 44 samples tested on the 50-gene panel 37 (84%) had a mutation in at least one of the three common mutated genes: *APC*, *TP53* and *KRAS* (Figure 5.1B). The small group that did not have one of these common mutations had a trend towards a poorer prognosis, with more disease recurrence (Figure 5.1C), although this did not reach statistical significance (p=0.065) with the small numbers available here. Mutations in *APC*, *TP53* and *KRAS* are well-known to have a role in driving the development of colorectal cancer. It is possible that tumours without these common mutations arise through an alternate pathway and this may have prognostic implications.

5.3.1.2 Tumour-Stroma Fraction

The tumour-stromal fraction, obtained by digital analysis at the Institute for Cancer Genetics and Informatics in Oslo, was available for 161 patients. The stromal fraction of the tumour varied from 19% to 74%, with a median of 35%. Examples of tumours with low and high stromal fraction are shown in Figures 5.2 & 5.3. A histogram for all samples is shown in Figure 5.4A.

In the literature a cut-off of 50% is often used to divide tumours into low and high stromal fraction[64]. Using this cut-off, 14 (8.7%) tumours were classified as high stroma. Figure 5.4B shows disease-free survival curves using this cut-off. High stroma tumours have a significantly higher risk

of recurrence with p = 0.02 and estimated 5-year disease-free survival of 48% compared with 77% for low stromal tumours.

A ROC curve was used to determine whether a different cut-off might be more suitable for this patient group (Figure 5.4C). This indicated an optimal cut-off at 38.2%, with AUC of 0.58. Using this cut-off 61 (38%) tumours were categorised as high stroma. Figure 5.4D shows the disease-free survival curves using the 38% cut-off. This gave a more significant difference between the groups, p=0.004, and estimated 5-year disease-free survival of 63% compared with 81% for low stromal tumours.

Although the tumour stromal fraction independently provided a moderately useful prognostic indicator of disease recurrence, when combined in a multivariate model including tumour pT stage, the stromal fraction was no longer significant. The two example cases depicted in Figures 5.2 & 5.3 provide some support to this, as the tumours are at the upper and lower extremes for stromal fraction (19% and 60%), but both were pT3 when excised, and both developed recurrent disease.

FIGURE 5.2 (OVERLEAF): EXAMPLE OF A TUMOUR WITH A LOW STROMAL FRACTION (19%). ALTHOUGH THE STROMAL FRACTION WAS LOW, THE TUMOUR WAS PT3. EXCISION WAS COMPLETE BUT THE PATIENT WAS DIAGNOSED WITH WIDESPREAD RECURRENCE 3.7 YEARS AFTER LATER. A. SCAN OF H&E SLIDE FROM THE TUMOUR. **B.** DIGITALLY PROCESSED SCAN SHOWING SEGMENTED STROMA IN LIGHT GREEN COLOUR. **C.** MASK OF TUMOUR PRODUCED BY DIGITAL PROCESSING, WITH STROMA IN GREEN. RED INDICATES TUMOUR CELLS AND BLUE IS ARTEFACT. THIS MASK WAS USED TO CALCULATE THE TUMOUR STROMAL FRACTION.

FIGURE 5.3 (P130): EXAMPLE OF A TUMOUR WITH A HIGH STROMAL FRACTION (60%). THE TUMOUR WAS PT3. THE PATIENT UNDERWENT ADJUVANT CHEMORADIOTHERAPY BUT WAS DIAGNOSED WITH MULTIPLE LIVER METASTASES 9 MONTHS AFTER LOCAL EXCISION. A. SCAN OF H&E SLIDE FROM THE TUMOUR. **B.** DIGITALLY PROCESSED SCAN SHOWING SEGMENTED STROMA IN LIGHT GREEN COLOUR. **C.** MASK OF TUMOUR PRODUCED BY DIGITAL PROCESSING, WITH STROMA IN GREEN. RED INDICATES TUMOUR CELLS AND BLUE IS ARTEFACT. THIS MASK WAS USED TO CALCULATE THE TUMOUR STROMAL FRACTION.

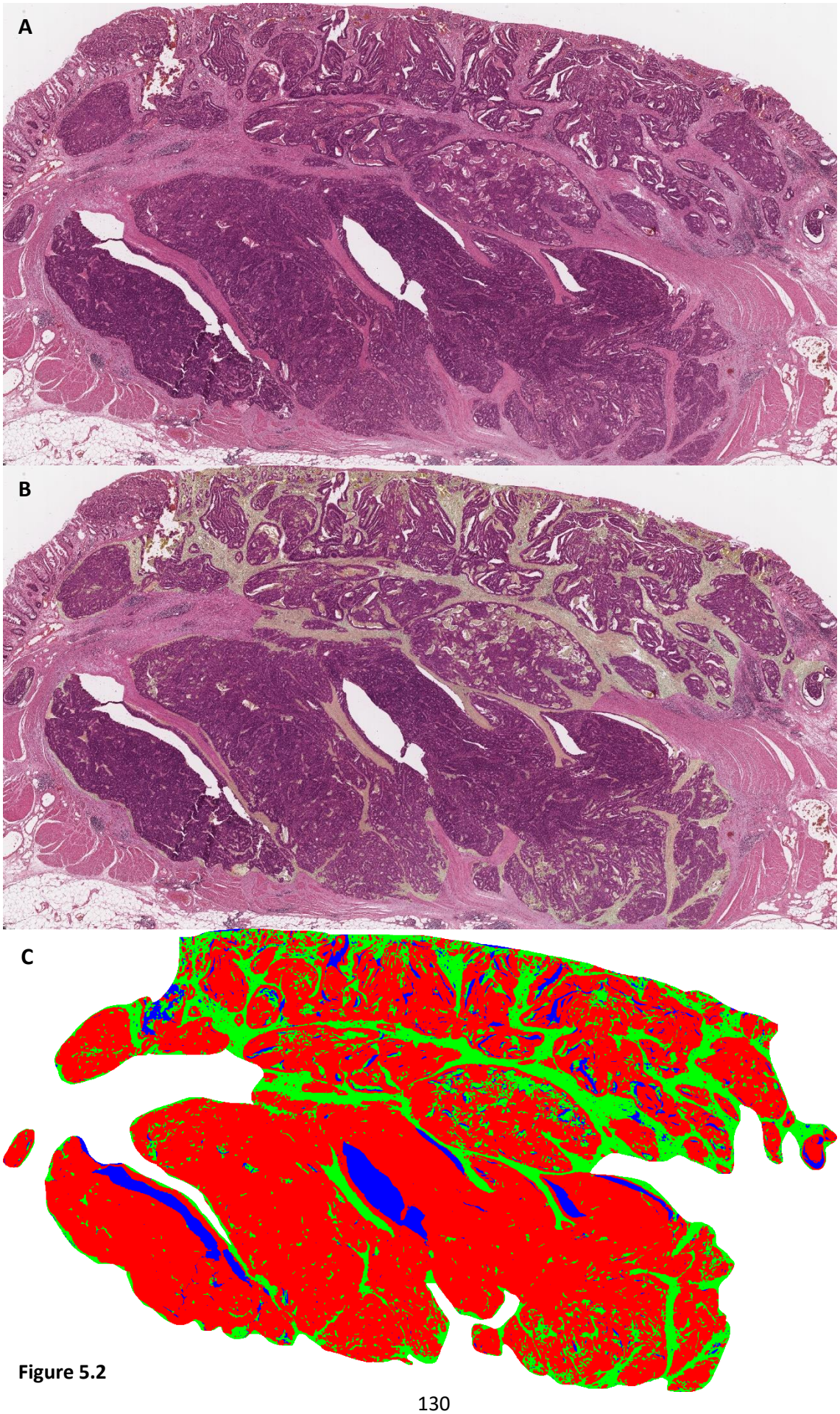


Figure 5.2

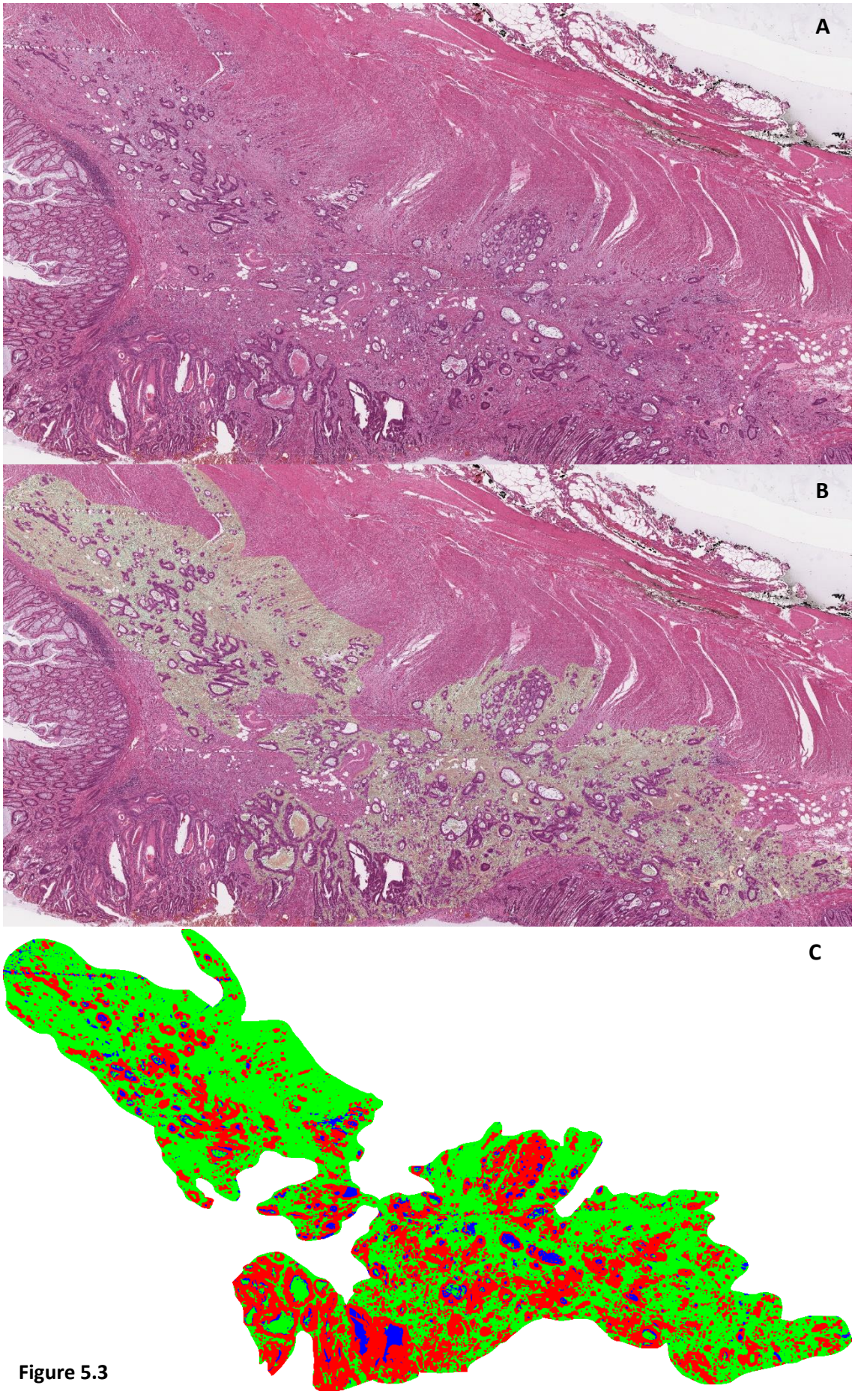


Figure 5.3

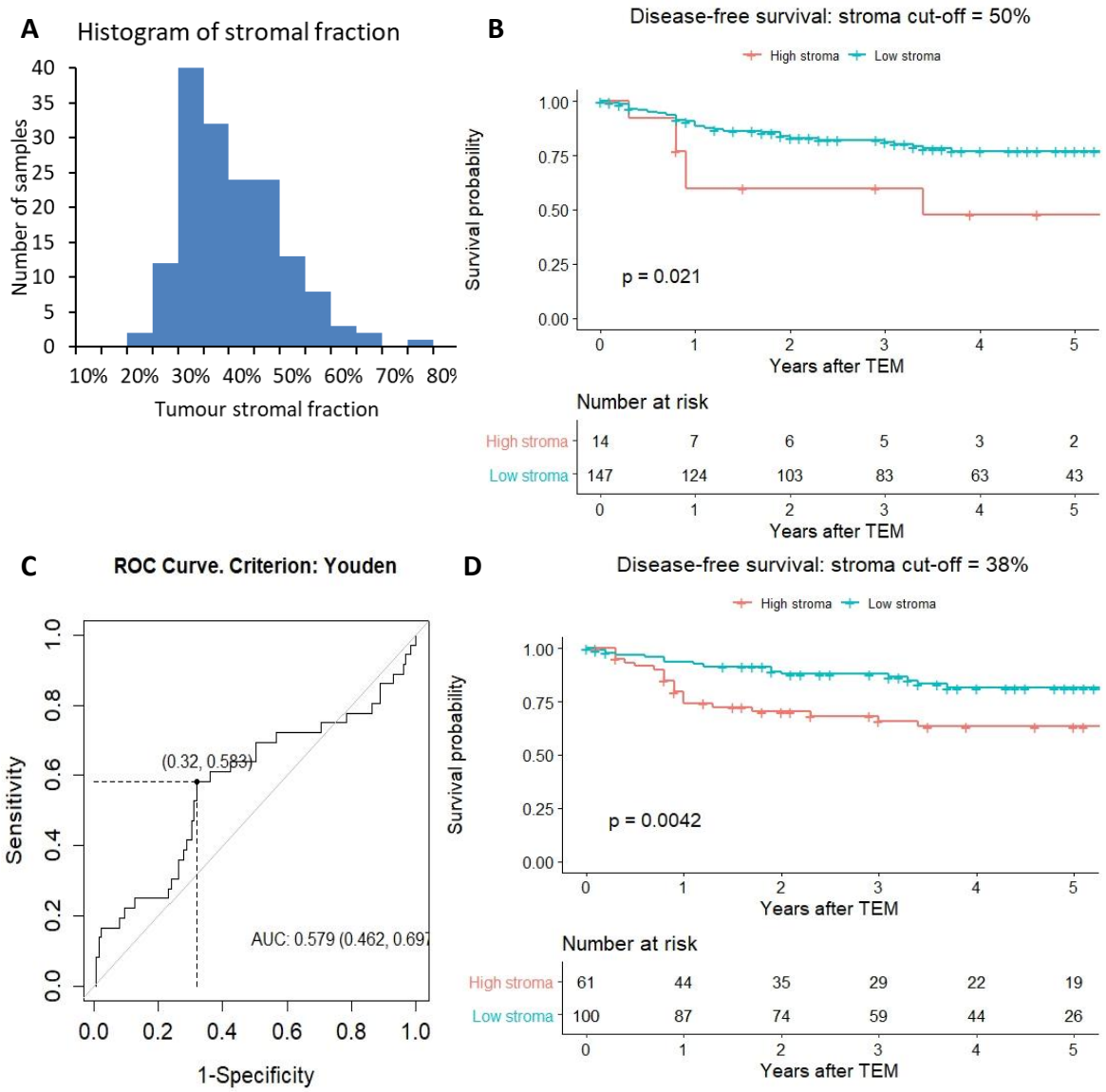


FIGURE 5.4: TUMOUR STROMAL FRACTION FOR 161 RECTAL CANCERS. A. HISTOGRAM OF TUMOUR STROMAL FRACTION. **B.** DISEASE-FREE SURVIVAL BY STROMAL FRACTION USING A CUT-OFF OF 50% STROMAL FRACTION. **C.** ROC CURVE ASSESSING STROMAL FRACTION AS PREDICTOR OF RECURRENCE. USING THE YOUDEN CRITERION, THE OPTIMAL CUT-OFF IS 38%, WITH AUC 0.58. **D.** DISEASE-FREE SURVIVAL BY STROMAL FRACTION USING A CUT-OFF OF 38%, AS DETERMINED BY THE ROC CURVE.

5.3.1.3 Digital pathology

Digital neural network (DNN) digital pathology results were available for 57 TEM cancer samples that had not had prior radiotherapy (four samples that failed quality control for digital analysis have been excluded). The tumour area was classified as epithelium, desmoplastic stroma, inflamed stroma, mesenchyme, mucin, and necrosis. The proportional areas are shown in Figure 5.5A. The

median proportion of epithelium in the tumour was 0.53 (interquartile range: 0.45-0.62), for desmoplastic stroma the median was 0.23 (IQR: 0.16-0.32) and for inflammatory stroma the median was 0.06 (IQR: 0.03-0.12).

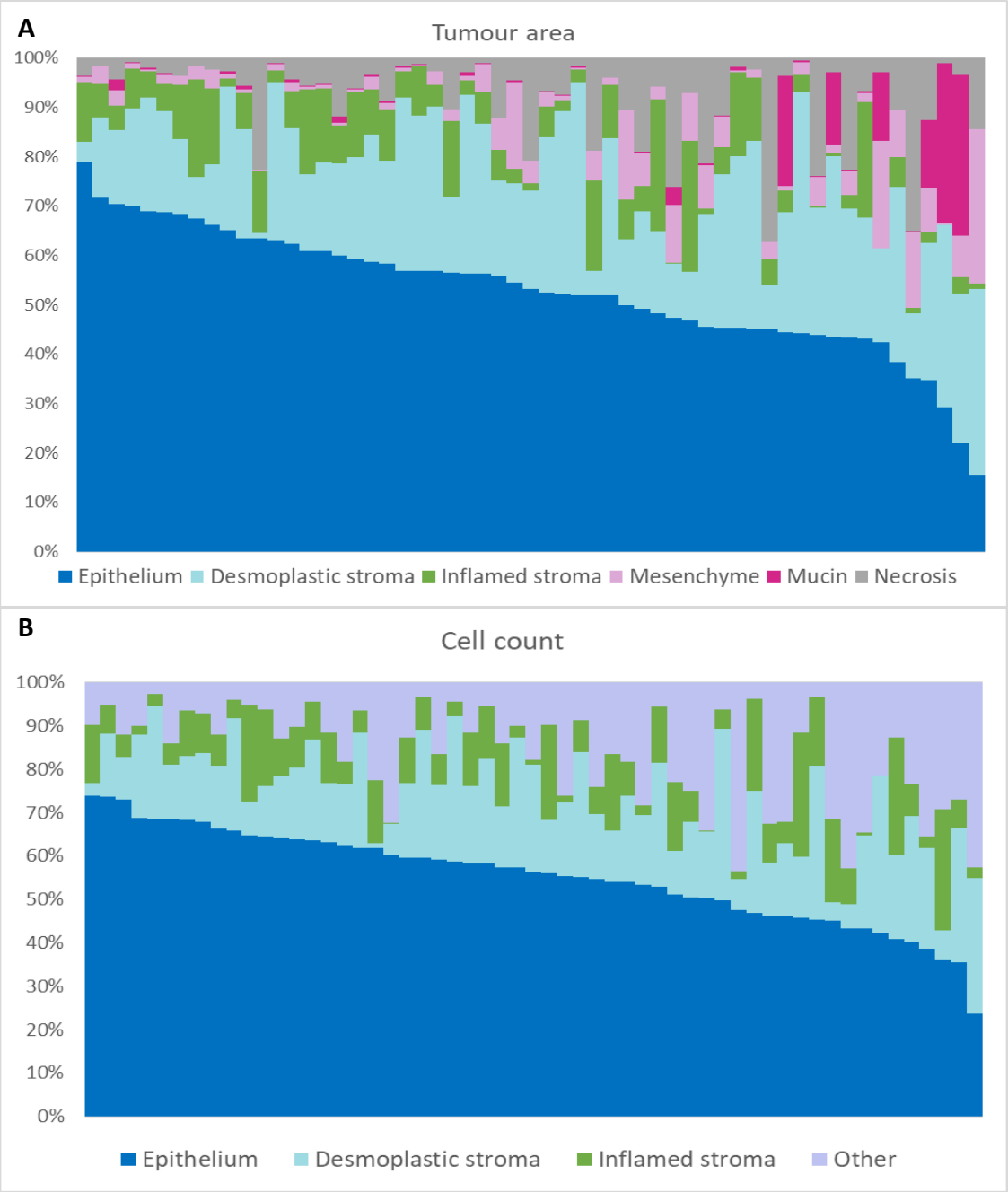


FIGURE 5.5: PROPORTIONAL AREA AND CELL COUNT OF THE TUMOUR COMPONENTS, DETERMINED BY DNN DIGITAL PATHOLOGY, IN 57 RECTAL CANCERS. IN MOST TUMOURS EPITHELIUM FORMS THE MAJOR PART AND EPITHELIAL CELLS PREDOMINATE. DESMOPLASTIC STROMA FORMS A SIGNIFICANT PART OF MOST TUMOURS WHILE INFLAMED STROMA MAKES UP A SMALLER, VARIABLE PROPORTION. FEW TUMOURS ARE MUCINOUS. MESENCHYME AND NECROSIS FORM ONLY A MINOR PART IN MOST TUMOURS. **A.** PROPORTIONAL AREA. **B.** PROPORTIONAL CELL COUNT.

The cells constituting the tumour total cell count were classified as epithelial, desmoplastic stroma, inflamed stroma and other. The proportional cell counts are shown in Figure 5.5B. The median proportion of epithelial cells in the tumour was 0.56 (IQR: 0.47-0.64), for desmoplastic stroma the median was 0.17 (IQR: 0.12-0.26) and for inflammatory stroma the median was 0.07 (IQR: 0.04-0.13).

Cellular density within the three tumour regions is shown in Figure 5.6. The mean cellular density for the whole tumour, excluding areas classified as necrosis, mucin, whitespace, mesenchyme, was 10,113 cells/mm^2 (standard deviation 2,049). Inflamed stroma was most dense, 11,082 cells/mm^2 (sd: 997) as expected being an inflammatory lymphocyte-rich infiltrate, followed by epithelium, 8,958 cells/mm^2 (sd: 1,239). The lowest cellular density was in the desmoplastic stroma, 6,734 cells/mm^2 (sd: 1,913), again as expected. This difference was significant (Kruskal-Wallis test, p value < 2.2 x 10^{-16}).

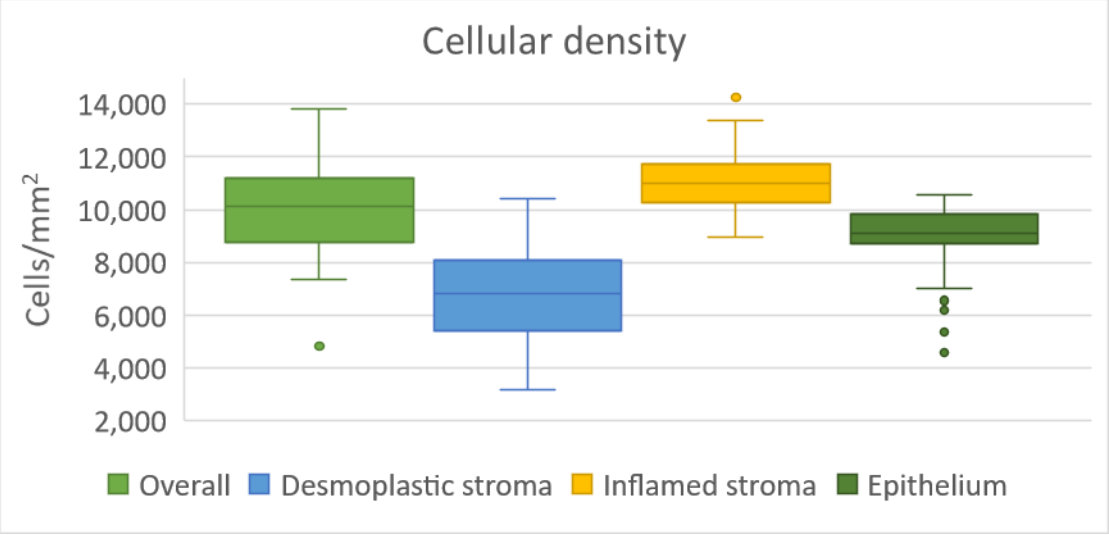


FIGURE 5.6: CELLULAR DENSITY IN THE THREE DEFINED TUMOUR REGIONS, ALSO SHOWING WHOLE TUMOUR CELLULAR DENSITY, DETERMINED BY DNN. DENSITY CALCULATED AS CELL COUNT DIVIDED BY AREA IN MM^2. INFLAMED STROMA HAS THE HIGHEST CELLULAR DENSITY AND DESMOPLASTIC STROMA, THE LOWEST.

The ratio of desmoplastic to inflamed (D:I) stromal area was calculated and compared with the occurrence of disease recurrence. Figure 5.7A shows the log of the ratio. Mann-Whitney testing

gives a p value of 0.015, indicating that, despite some overlap, there is a significant difference in D:I values for patients who do and do not develop disease recurrence after TEM, with more desmoplastic stroma relative to inflamed stroma in those who get recurrent disease. A similar pattern was obtained when considering the ratio of cell count.

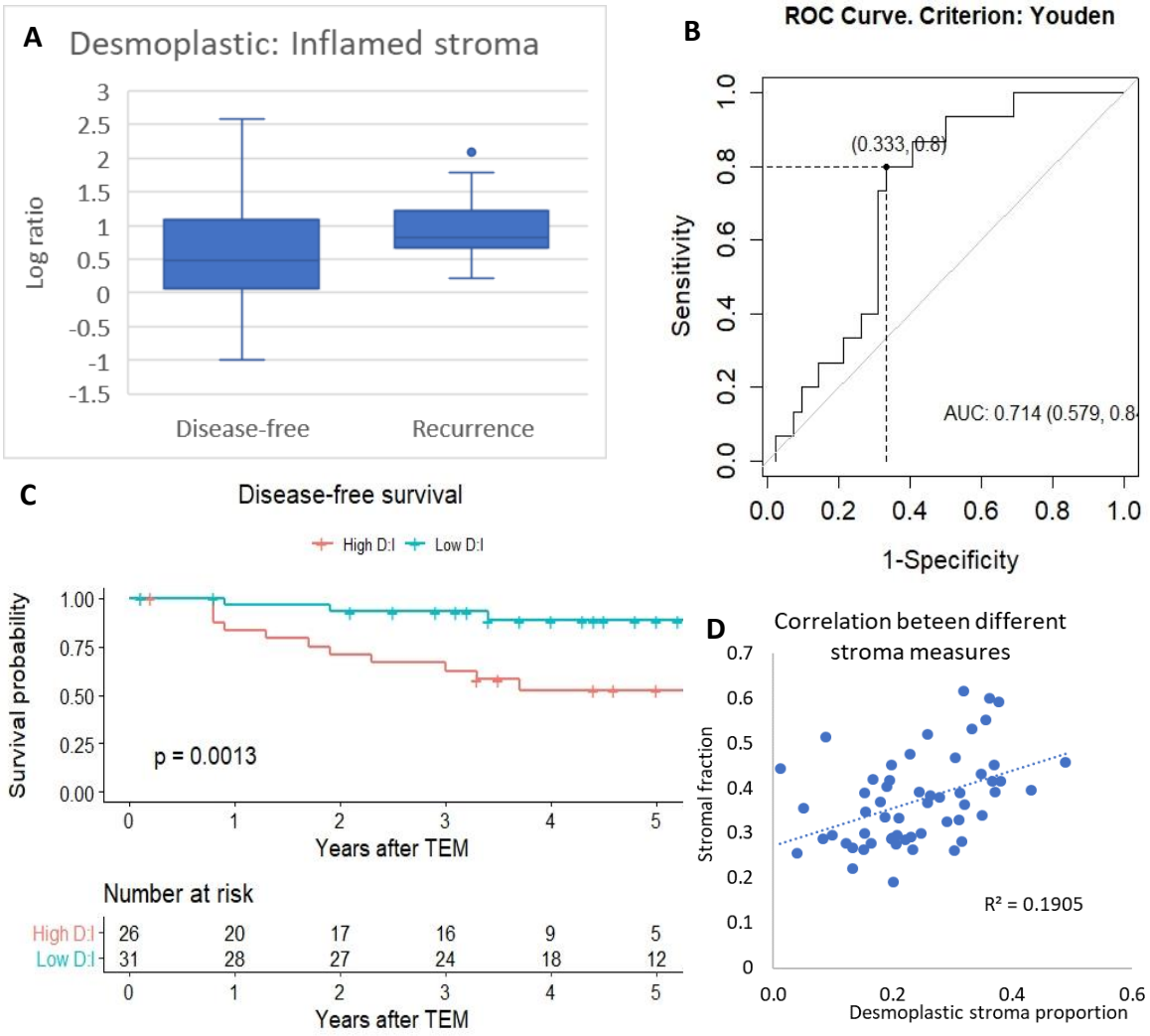


FIGURE 5.7: LOG RATIO OF DESMOPLASTIC TO INFLAMED STROMA AREA IN 57 RECTAL CANCERS. A: BOX AND WHISKER PLOT SHOWING LOG D:I FOR STROMAL AREA IN 57 RECTAL CANCERS. THE MEDIAN LOG D:I WAS 0.82 IN THE 15 WHO DEVELOPED RECURRENCE AND 0.48 IN THE 42 WHO REMAINED DISEASE FREE. LOG D:I WAS SIGNIFICANTLY HIGHER IN THE RECURRENCE GROUP, P=0.015. **B**: ROC CURVE ASSESSING LOG D:I AS PREDICTOR OF RECURRENCE. USING THE YOUDEN CRITERION, THE OPTIMAL CUT-OFF IS LOG D:I = 0.67, WITH AUC 0.71. **C.** DISEASE-FREE SURVIVAL BY D:I, WITH HIGH D:I DEFINED AS ≥ 4.67, THE CUT-OFF VALUE ON ROC CURVE. **D.** SCATTER PLOT SHOWING CORRELATION OF DESMOPLASTIC STROMA PROPORTION (DETERMINED BY DNN) WITH TUMOUR STROMAL FRACTION (DETERMINED BY A SIMPLER DIGITAL ANALYSIS).

Assessing the value of the D:I ratio as a predictor of recurrence produced the ROC curve in Figure 5.7B. This chose a cut-off for the ratio at 4.67 (or 0.67 for the log ratio).

The area under the curve is 0.71 (95% CI: 0.58-0.85). At this cut-off, the sensitivity is 0.80, specificity 0.67, PPV 0.46 and NPV 0.90. This model defined a poor prognosis group with high D:I comprising 46% (26/57) of the total. The disease-free survival curve shows a significant survival advantage in those with a low ratio (Figure 5.11C). The 5-year disease-free survival for the high D:I group is 53% (95% CI: 35-78) compared with 89% (95% CI: 77-100) for the low D:I group.

Once the DNN has been trained, these values can be quickly and simply obtained from an H&E stained slide of the tumour. So, although PPV is not high, the D:I ratio may have a supplemental role in predicting recurrence risk for a patient. The proportional tumour area of desmoplastic stroma obtained via DNN methodology was compared with the stromal fraction described in the previous subsection. Figure 5.11D shows only a weak correlation, $R^2 = 0.19$.

MOLECULAR SCORING FOR TUMOUR COMPARTMENTS

An alternative to digital pathology to infer the fraction of stromal and immune cells in a tumour is the ESTIMATE method[101] which uses gene expression data for 141 stromal and 141 immune genes. The sum of the stromal and immune scores gives the ESTIMATE score which is used to give a measure of tumour purity. These scores were compared with the occurrence of disease recurrence, but there was no significant association for either stromal or immune score, nor for a ratio of the two. Results for the scores were compared to those obtained with digital pathology. Figure 5.8 shows the correlation of digital pathology with ESTIMATE scores, comparing the desmoplastic stroma cell count proportion with the ESTIMATE stromal score (A), and the inflamed stroma cell count proportion with the ESTIMATE immune score (B). These showed a modest correlation, with the relatively small number of samples. The lack of strong correlation may account for the failure of the scores to show an association with disease recurrence.

ESTIMATE scores were also compared with the proportional area for each compartment, and these correlations were weaker than for the cell count proportion, as may be expected. The ESTIMATE stromal score was also compared with the tumour stromal fraction obtained in the previous section and showed only a weak correlation with $R^2 = 0.17$ (Figure 5.8C).

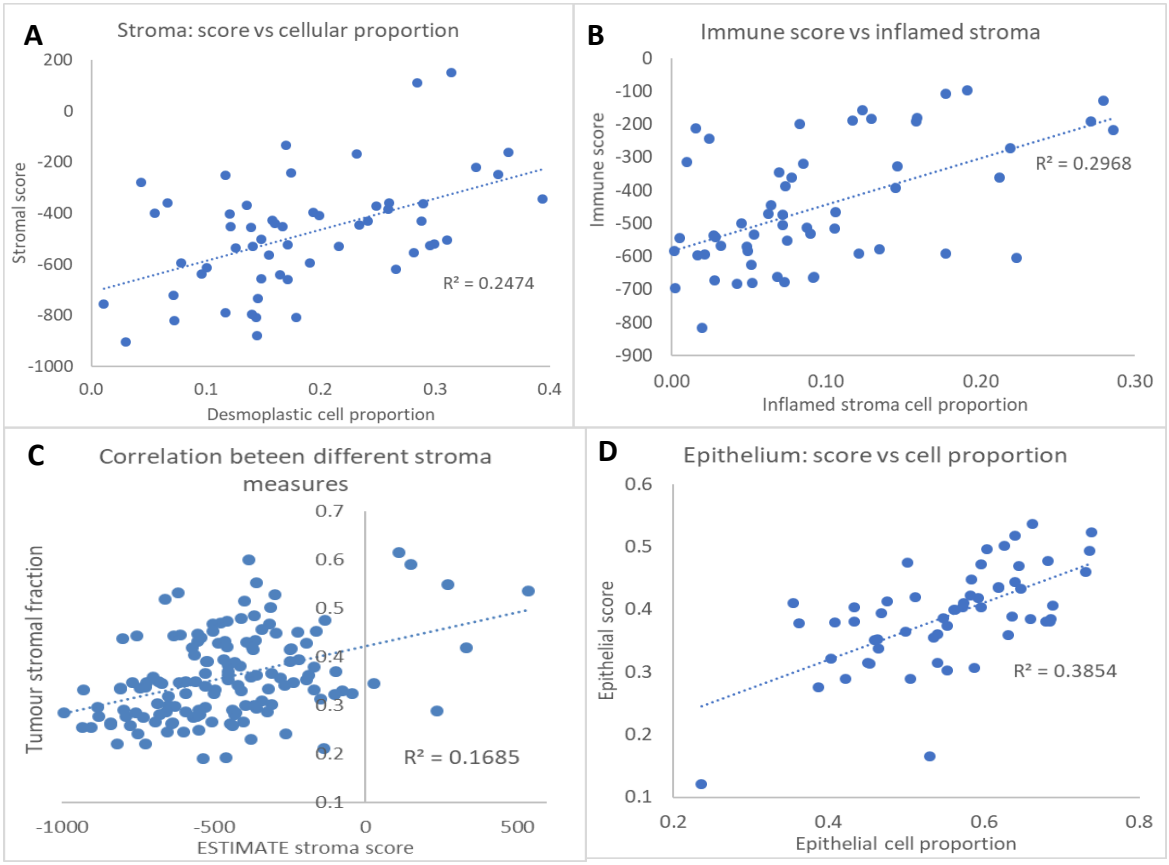


FIGURE 5.8: SCATTER PLOTS SHOWING CORRELATION BETWEEN DIGITAL PATHOLOGY AND GENE EXPRESSION SCORES FOR 57 RECTAL CANCERS. A. COMPARISON OF ESTIMATE STROMAL SCORE WITH THE CELL COUNT PROPORTION OF DESMOPLASTIC STROMA CELLS DETERMINED BY DNN IN 57 RECTAL CANCERS, $R^2 = 0.247$. **B.** COMPARISON OF ESTIMATE IMMUNE SCORE WITH THE CELL COUNT PROPORTION OF INFLAMED STROMA CELLS DETERMINED BY DNN IN 57 CANCER, $R^2 = 0.297$. **C.** SCATTER PLOT SHOWING CORRELATION OF ESTIMATE STROMA SCORE WITH TUMOUR STROMAL FRACTION. **D.** COMPARISON OF EPITHELIAL SIGNATURE SCORE[103] CALCULATED USING SSGSEA METHOD, WITH PROPORTION OF EPITHELIAL CELLS IN THE TUMOUR, DETERMINED BY DNN, $R^2 = 0.385$.

An epithelial signature score was also calculated for all samples using Tan's list of epithelial genes[103]. A comparison of the epithelial signature score with the proportion of epithelial cells in the tumour on digital pathology is also shown in Figure 5.8D. This has R^2 value of 0.39, higher than for either stromal component. A comparison of epithelial score with digital proportional area of

epithelium was also assessed, and again the correlation was weaker than with cell count proportion.

The ESTIMATE tumour purity score was compared to the epithelial signature score for all 150 samples that had not had prior radiotherapy, and to the digitally determined epithelial area proportion of the tumour for the 57 samples with DNN pathology data. These comparisons are shown in Figure 5.9 where it can be seen that for epithelial score there is a strong correlation (R^2=0.71), while for epithelial area proportion the correlation is much weaker (R^2=0.27), although this may partly reflect a much smaller number of samples. Overall, these three measures are reasonably consistent.

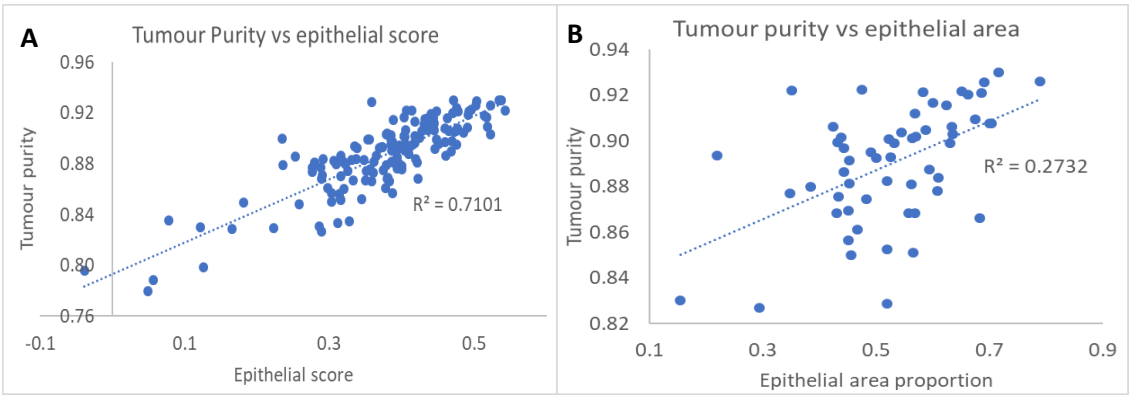


FIGURE 5.9: SCATTER PLOTS SHOWING TUMOUR PURITY AND EPITHELIAL SCORE AND AREA. A. TUMOUR PURITY SHOWS STRONG CORRELATION WITH EPITHELIAL SCORE DETERMINED FROM GENE EXPRESSION DATA (SSGSEA METHOD) FOR 151 RECTAL CANCERS, R^2 = 0.71. **B.** THERE IS A WEAKER CORRELATION BETWEEN TUMOUR PURITY AND EPITHELIAL PROPORTIONAL AREA OF THE TUMOUR AS DETERMINED BY DIGITAL PATHOLOGY IN A SUBSET OF 57 RECTAL CANCERS, R^2 = 0.27.

The ratio of the ESTIMATE stromal score to immune score was calculated and compared with the occurrence of disease recurrence, to see whether the association seen with the DNN desmoplastic to inflamed stroma ratio was replicated. However, with the ESTIMATE scores there was no significant difference between the groups (Mann-Whitney test, p = 0.06).

There were few mucinous cases among the samples with digital pathology, so it was not possible to compare a relevant gene expression signature with mucin area.

DNN NUCLEAR FEATURES

For cells in the epithelium and desmoplastic and inflamed stroma, DNN digital pathology measurement was also made of the average nuclear optical density, average nucleus area and average nucleus perimeter. These are shown in Figure 5.10.

The optical density provides a measure of the chromatin content. The average nuclear optical density does not show any significant difference between the three cell types (Kruskal-Wallis test, p value 0.33). One tumour is an outlier in each category with very high optical density; there were no other unusual features about this tumour or patient, so this is most likely due to staining artefact or increased section thickness.

The area and perimeter of the nucleus are correlated but the perimeter measure shows increased variance when the contour of the nucleus is more irregular. Figure 5.10B plots the average perimeter against area for nuclei in the three compartments of each sample. Epithelial cell nuclei are larger than those in the stromal cells. The desmoplastic stromal cell nuclei, which are mostly cancer-associated fibroblasts (CAF), have a larger perimeter relative to area than the inflamed stromal cell nuclei (mostly lymphocytes) indicating less rounded, more irregular nuclei in the desmoplastic stroma. The correlation between nuclear area and perimeter for the desmoplastic cellular nuclei is also less than for the other two compartments (R^2 = 0.94 vs 0.97), again indicating a little more variability in the nuclei contour.

The relative difference between the average nuclear area and perimeter showed a significant difference between the compartments (Kruskal-Wallis test, p value < 2.2×10^{-16}) due to the perimeter of epithelial nuclei being less than the area, reflecting the larger size. A non-significant association with prognosis was seen in the inflamed stroma component, p = 0.052; the group with recurrence tended to have smaller nuclear area in the inflamed stroma cells than the disease-free group which may reflect more reactive or B lymphocytes in the better prognosis group.

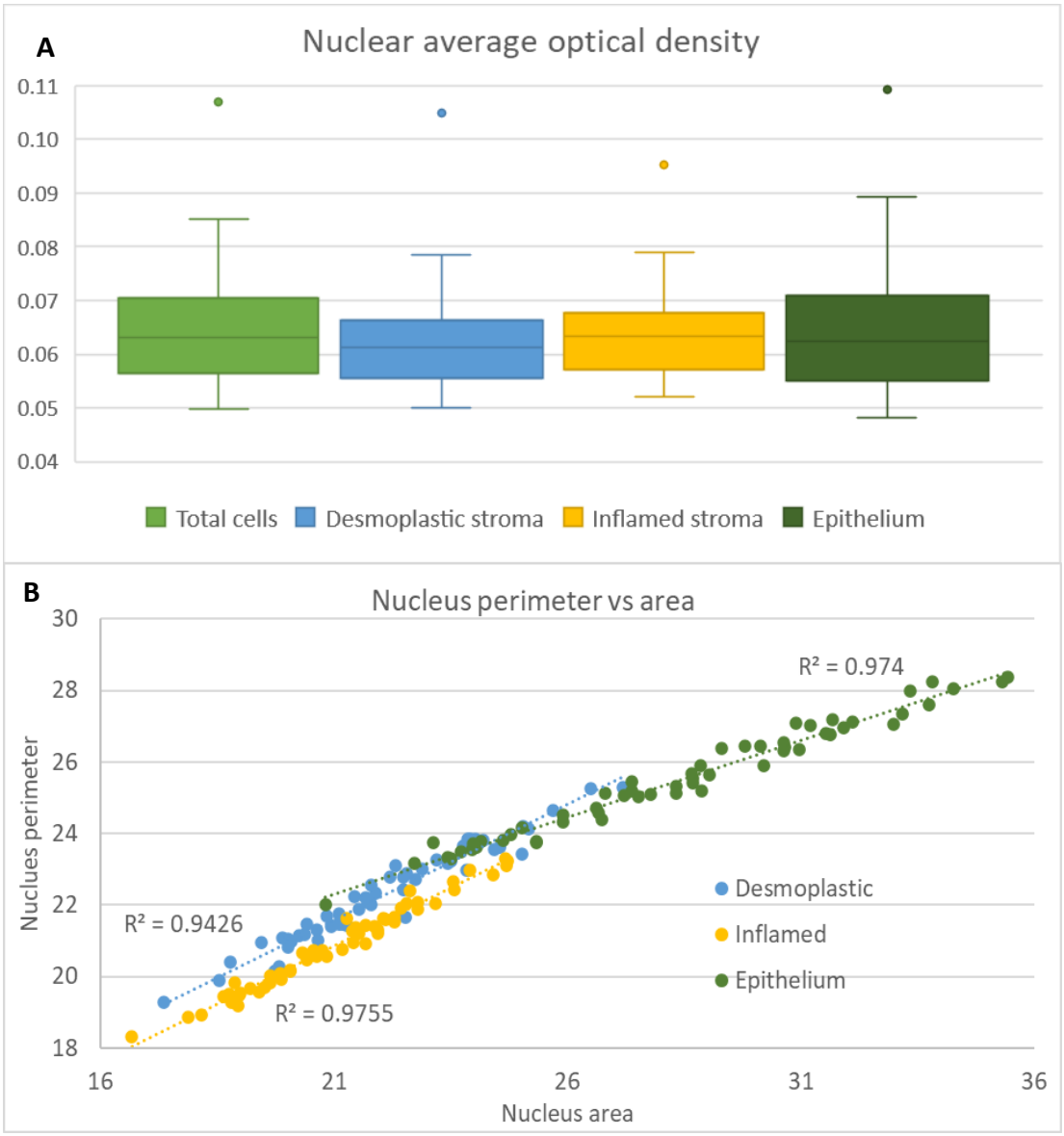


FIGURE 5.10: NUCLEAR FEATURES OF THE COMPONENTS OF 57 RECTAL CANCERS, DETERMINED BY DNN. A: AVERAGE NUCLEAR OPTICAL DENSITY, DETERMINED BY HAEMATOXYLIN STAINING INTENSITY, FOR ALL TUMOUR CELLS, AND FOR CELLS IN THE THREE MAIN TUMOUR COMPARTMENTS, INDICATING NO DIFFERENCE BETWEEN COMPARTMENTS. **B**: SCATTER PLOT OF AVERAGE NUCLEAR PERIMETER AGAINST AVERAGE NUCLEAR AREA FOR THE THREE COMPARTMENTS. THE TREND LINE FOR EACH COMPARTMENT IS LINEAR AND SHOWS THE R^2 VALUE.

NUCLEOTYPING

An assessment of DNA organisation within the tumour epithelial cell nuclei was made using image texture analysis at the Institute for Cancer Genetics and Informatics in Oslo. Results of this nucleotyping were available for 156 samples, including 55 where the nuclear optical density and

average nucleus area and perimeter were also available. Based on earlier studies in multiple cancer types including colorectal[51], a cut-off chromatin value of 0.044 was determined as differentiating chromatin homogenous (better prognosis) from chromatin heterogenous (worse prognosis) tumours. Using this cut-off, 36 (23%) tumours were classified as chromatin heterogeneous; disease-free survival was no different between the groups (Figure 5.11A).

A ROC curve was used to assess whether an alternative cut-off may be better in the current population. The optimal cut-off determined by the data was 0.028, although AUC was only 0.52. Using this cut-off, 25 (16%) tumours were classified as chromatin heterogeneous. Although the groups were more distinct the disease-free survival was not significantly different, and the chromatin heterogenous group tended to have better survival, contrary to expectation (Figure 5.11C). The chromatin values were compared with the nuclear optical density determined by DNN, also a measure of chromatin content, for epithelial cells (Figure 5.11D) and no association was detected, $R^2 = 0$.

DNA PLOIDY STATUS

The DNA ploidy status was assessed by image cytometry at the Institute for Cancer Genetics and Informatics in Oslo. Results were available for 158 tumours. The majority of tumours were aneuploid, (103, 65%), while 14 (9%) were tetraploid and 41 (26%) were diploid. Non-diploid tumours are generally considered to have poorer prognosis. Figure 5.12 shows examples of a diploid (A) and an aneuploid (B) tumour. Also shown are disease-free survival curves by ploidy status. For this population, there was no difference in survival according to ploidy status. However, when combined with tumour-stromal fraction using a 50% cut-off, following Danielsen *et al*[64], those with nondiploid and high stromal tumours had significantly worse disease-free survival than the other groups (Figure 5.12D).

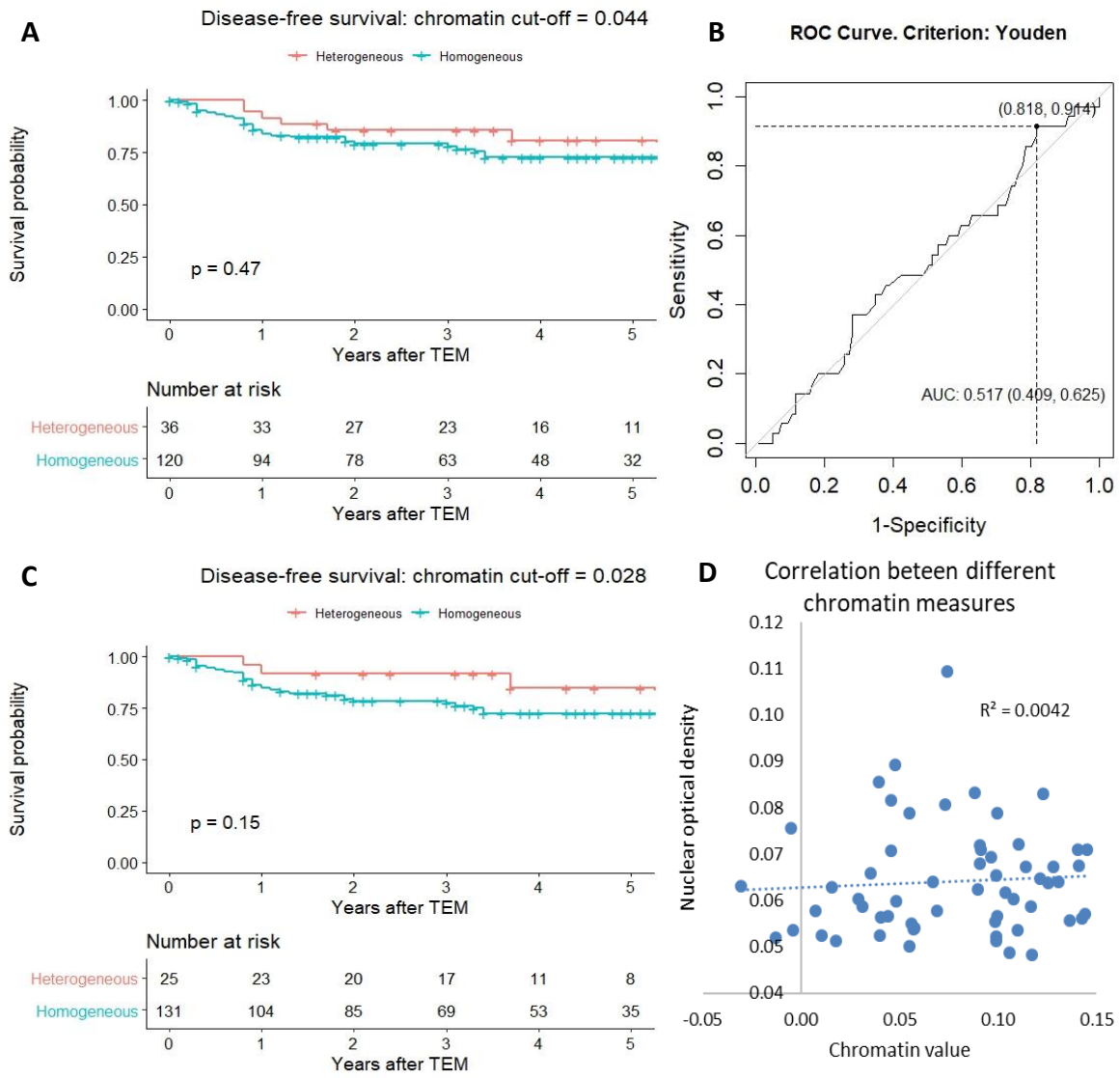


FIGURE 5.11: CHROMATIN ORGANISATION FOR 156 RECTAL CANCERS. A. DISEASE-FREE SURVIVAL BY CHROMATIN HETEROGENEITY USING A CUT-OFF OF 0.044 CHROMATIN VALUE, SHOWING NO DIFFERENCE BETWEEN GROUPS. **B.** ROC CURVE ASSESSING CHROMATIN VALUE AS PREDICTOR OF RECURRENCE. USING THE YOUDEN CRITERION, THE OPTIMAL CUT-OFF IS 0.028, WITH AUC 0.52. **C.** DISEASE-FREE SURVIVAL BY CHROMATIN HETEROGENEITY USING A CUT-OFF OF 0.028, AS DETERMINED BY THE ROC CURVE. **D.** SCATTER PLOT SHOWING CORRELATION OF CHROMATIN VALUE WITH AVERAGE NUCLEAR OPTICAL DENSITY OF TUMOUR EPITHELIAL CELLS.

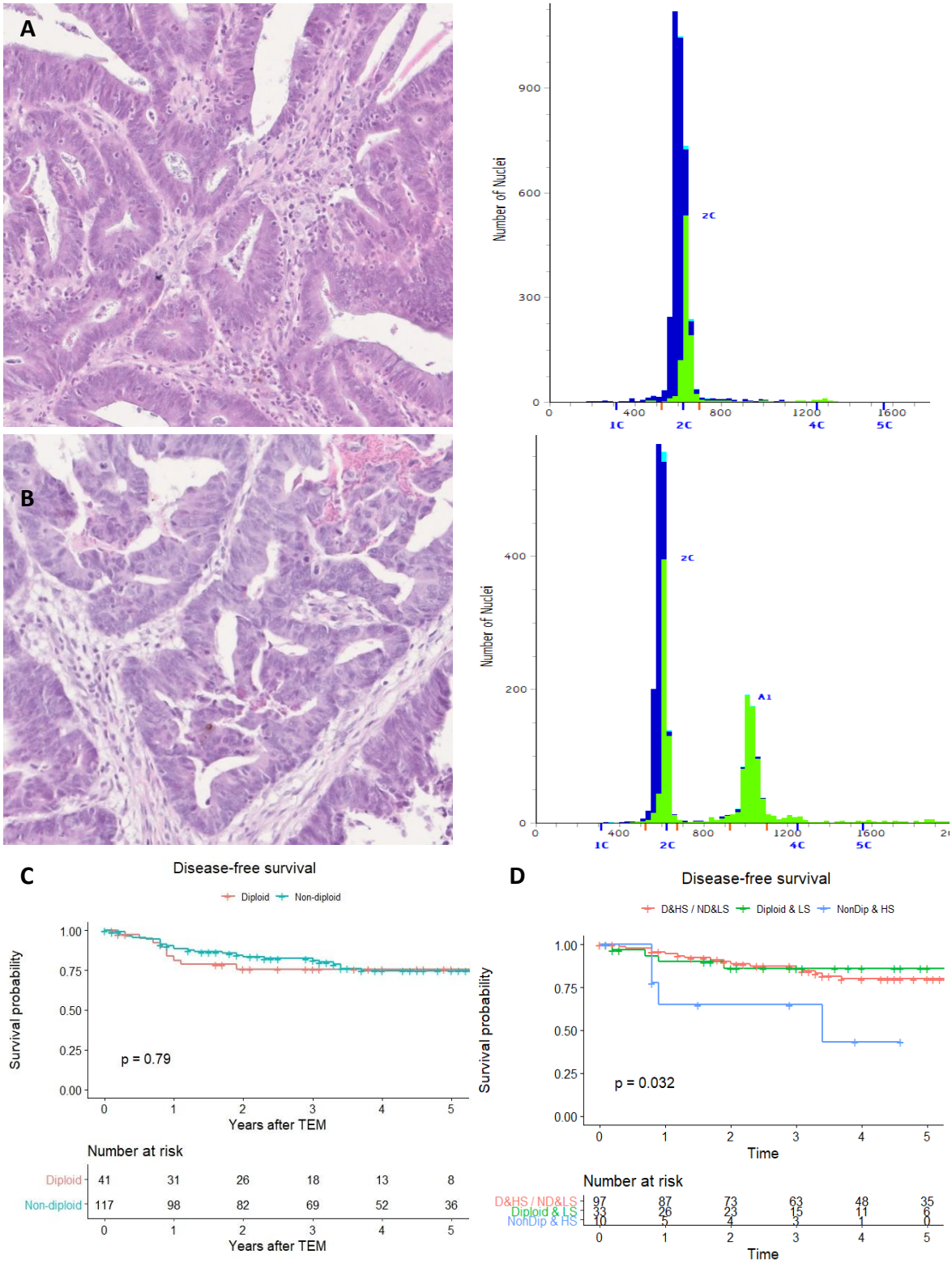


FIGURE 5.12: DNA PLOIDY STATUS AND DISEASE-FREE SURVIVAL. A. HISTOLOGY AND DNA HISTOGRAM OF TUMOUR WITH A DIPLOID HISTOGRAM, PLOTTING NUMBER OF NUCLEI AGAINST INTEGRATED OPTICAL DENSITY. **B.** HISTOLOGY AND DNA HISTOGRAM OF TUMOUR WITH AN ANEUPLOID HISTOGRAM. **C.** DISEASE-FREE SURVIVAL BY PLOIDY STATUS FOR 158 RECTAL CANCERS, SHOWING NO DIFFERENCE BETWEEN GROUPS. **D.** DFS BY COMBINED PLOIDY AND TUMOUR-STROMAL FRACTION. D&HS/ND&LS = DIPLOID & HIGH STROMA + NONDIPLOID & LOW STROMA, LS = LOW STROMA, NONDIP = NONDIPLOID, HS = HIGH STROMA.

5.3.1.4 Molecular subtypes

The molecular classification was assessed for 168 samples. The CMS package classified 17 (10%) as CMS1, 30 (18%) as CMS2, 32 (19%) as CMS3 and 46 (27%) as CMS 4; 43 (26%) could not be classified. The CRIS package using the top scoring pair algorithm (TSP) method scored 49 (29%) as CRIS-A, 7 (4%) as CRIS-B, 58 (35%) as CRIS-C, 26 (16%) as CRIS-D and 19 (11%) as CRIS-E; 9 (5%) were unclassified. Figure 5.13 shows the proportional classification of these tumours compared with the original published descriptions for colorectal cancers[74,76]. Overall, the rectal cancer set showed fewer CMS 1 and 2, and more CMS 3 and 4 than the original paper, and more CRIS A, C and D with fewer CRIS-B than the original.

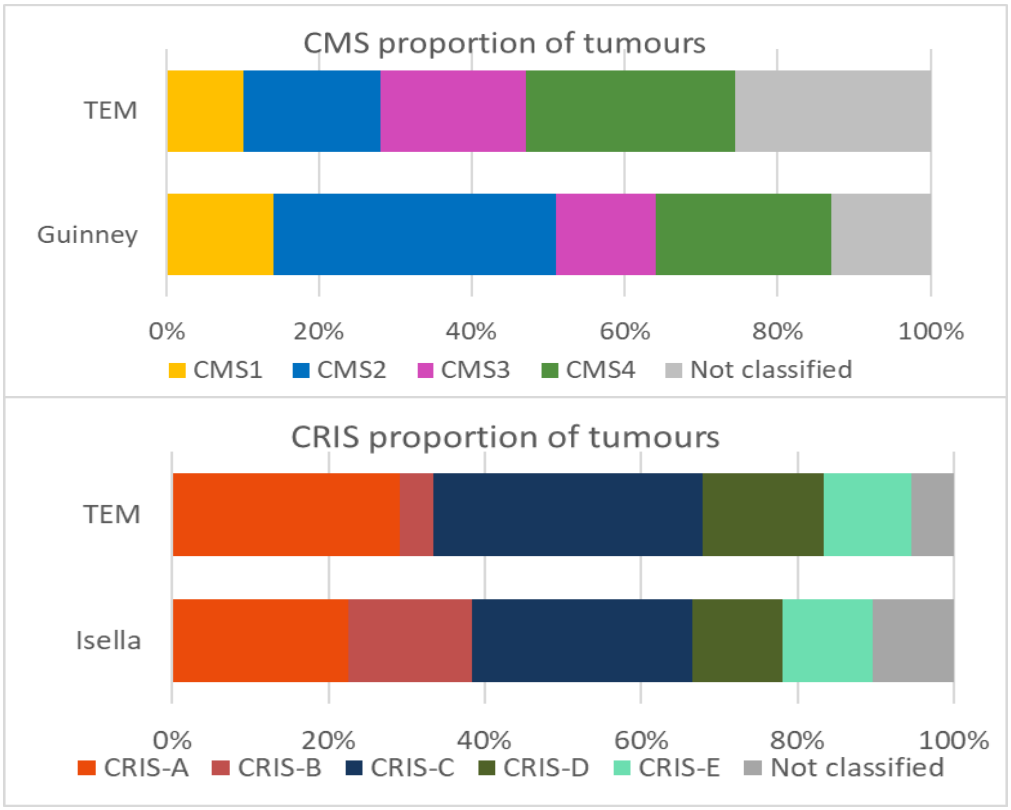


FIGURE 5.13: MOLECULAR SUBTYPES FOR 168 RECTAL CANCERS AND PUBLISHED COLORECTAL SERIES. THE PROPORTION OF TEM SERIES RECTAL CANCERS IN THE CMS AND CRIS SUBTYPES ARE COMPARED WITH THE PROPORTIONS FOUND IN PUBLISHED SERIES OF COLORECTAL CANCERS, GUINNEY *ET AL.*[74] FOR CMS AND ISELLA *ET AL.*[76] FOR CRIS.

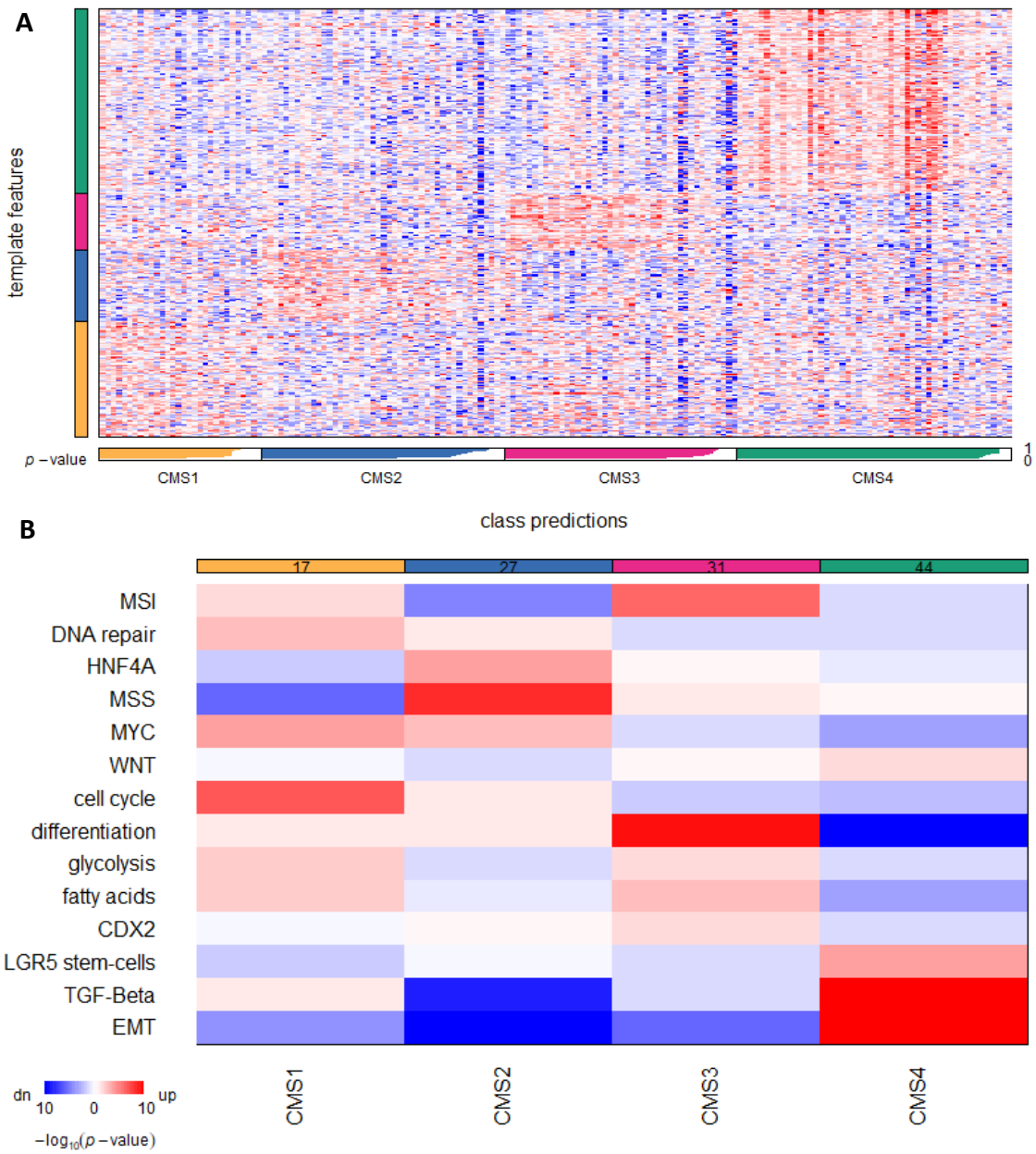


FIGURE 5.14: CMS SUBTYPES OF RECTAL CANCERS. A. HEATMAP SHOWING RELATIVE EXPRESSION FOR CMS TEMPLATE GENES FOR 125 CLASSIFIED SAMPLES. THE SAMPLES, IN COLUMNS, ARE ORDERED ACCORDING TO CLASS PREDICTIONS AND CONFIDENCE. THE HEIGHT OF THE WHITE BARS IN THE AXIS KEY GIVES THE UNADJUSTED PREDICTION P-VALUES. COLOUR SATURATION ON THE PLOT INDICATES MAGNITUDE; RED INDICATES OVER-EXPRESSION (ABOVE SAMPLE MEAN) AND BLUE INDICATES UNDER-EXPRESSION. **B.** PLOT SHOWING CAMERA GENE SET ANALYSIS FOR THE 118 SAMPLES THAT COULD BE CLASSIFIED USING THE 14,233 ENTREZ-IDENTIFIED GENES. COLOUR SATURATION INDICATES STATISTICAL SIGNIFICANCE.

The heatmap in Figure 5.14A shows the relative expression for CMS template genes for the 125 classified samples. The related gene set analysis was done using the 118 samples that could be classified using the 14,233 Entrez-identified genes. Figure 5.14B shows the results for Camera gene

set analysis. The gene set analysis of the CMS subtypes generally shows the expected patterns. For CMS1 there is over-expression of cell cycle genes and low expression of microsatellite stability genes. For CMS2 the microsatellite stability genes are over-expressed whereas EMT and TGF-β genes show very low expression. CMS2 also showed some increase in Myc genes but not in Wnt genes. In CMS3 the differentiation genes are highly expressed and EMT genes are under-expressed, consistent with epithelial and metabolic dysregulation. In CMS4, in contrast to CMS2, EMT and TGF-β genes are strongly over-expressed, while differentiation genes have low expression.

A heatmap for the CRIS classification is shown in Figure 5.15. There is a reddish tinge to the cells on the main diagonal but it is not as convincing as the CMS heatmap.

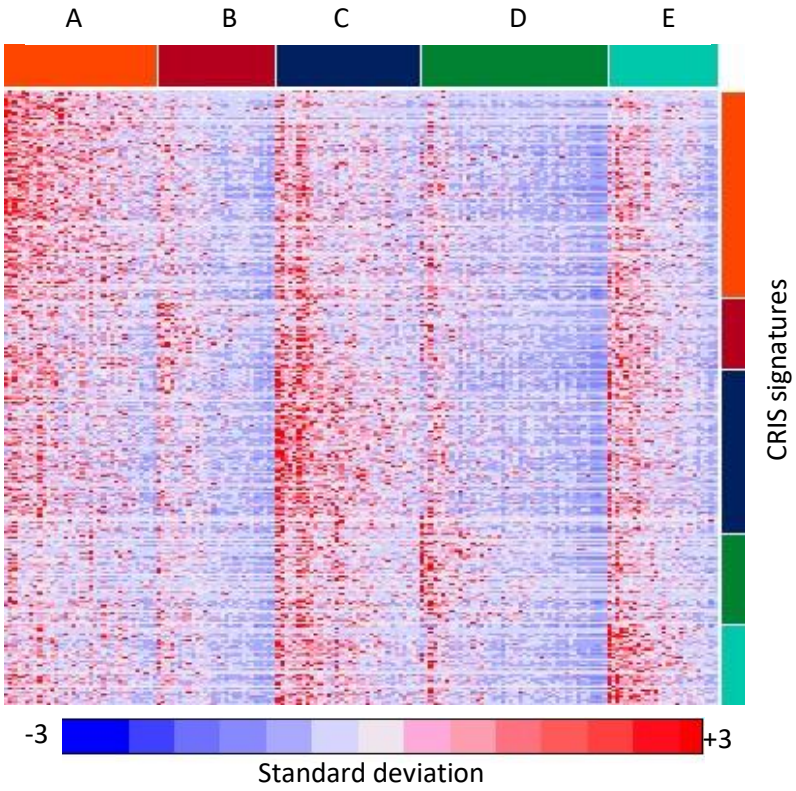


FIGURE 5.15: HEATMAP FOR CRIS SUBTYPES FOR 168 RECTAL CANCERS. THIS HEATMAP USED THE NEAREST TEMPLATE PREDICTOR (NTP) METHOD.

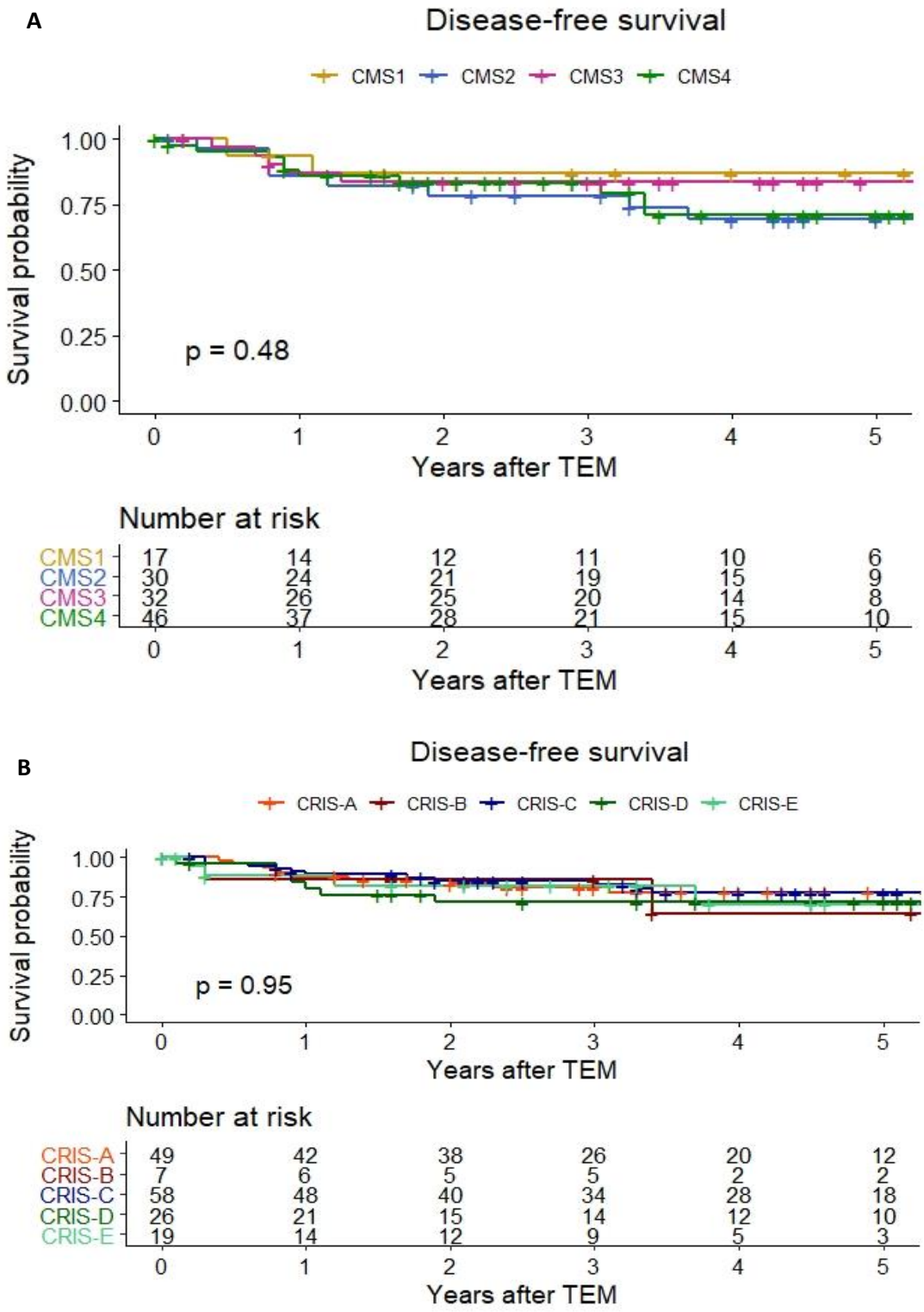


FIGURE 5.16: DISEASE-FREE SURVIVAL BY MOLECULAR SUBTYPE FOR CLASSIFIED RECTAL CANCERS. A. BY CMS FOR 125 SAMPLES: CMS 1 AND 3 TENDED TO HAVE LESS RECURRENCE THAN CMS 2 AND 4 IN THE LONGER TERM, BUT THE DIFFERENCES WERE NOT SIGNIFICANT. **B.** BY CRIS FOR 159 SAMPLES. THERE IS NO DIFFERENCE IN THE GROUPS.

Disease-free survival by subtype is shown in Figure 5.16. For CMS, this shows similar risk of recurrence till about 3 years following surgery, then the CMS2 and CMS 4 groups have a higher disease recurrence rate, although the differences are not significant. In Guinney *et al.*'s paper[74] CMS4 has significantly worse disease-free survival; there is a non-significant trend in this direction here.

In Isella *et al.*'s paper[76] CRIS-B has a significantly poorer disease-free survival but no such effect was seen in the present series, although CRIS-B was rare (4% vs 16% in Isella *et al*.).

COMPARISON WITH DIGITAL PATHOLOGY AND MOLECULAR SCORING

The CMS classification was available for 42 of the samples which had been classified by DNN digital pathology. Figure 5.17 shows a comparison of the desmoplastic to inflamed stromal area and the proportional epithelial area by CMS group. The differences between CMS groups are not significant: for D:I ratio the Kruskal-Wallis test gives p value 0.096 and for epithelial proportion the p value is 0.078. However, a comparison of CMS1 with CMS4 showed a significantly higher D:I ratio for CMS4 ($p = 0.023$, Mann-Whitney test), the mesenchymal subtype with strong TGF-β expression compared with CMS1, associated with strong immune activation.

The CRIS classification was available for 52 of the samples with DNN digital pathology; there was no significant association between CRIS type and the desmoplastic to inflamed stroma ratio or the epithelial proportion of the tumour; this is not surprising as the CRIS signature is designed to ignore the stromal component of the tumour.

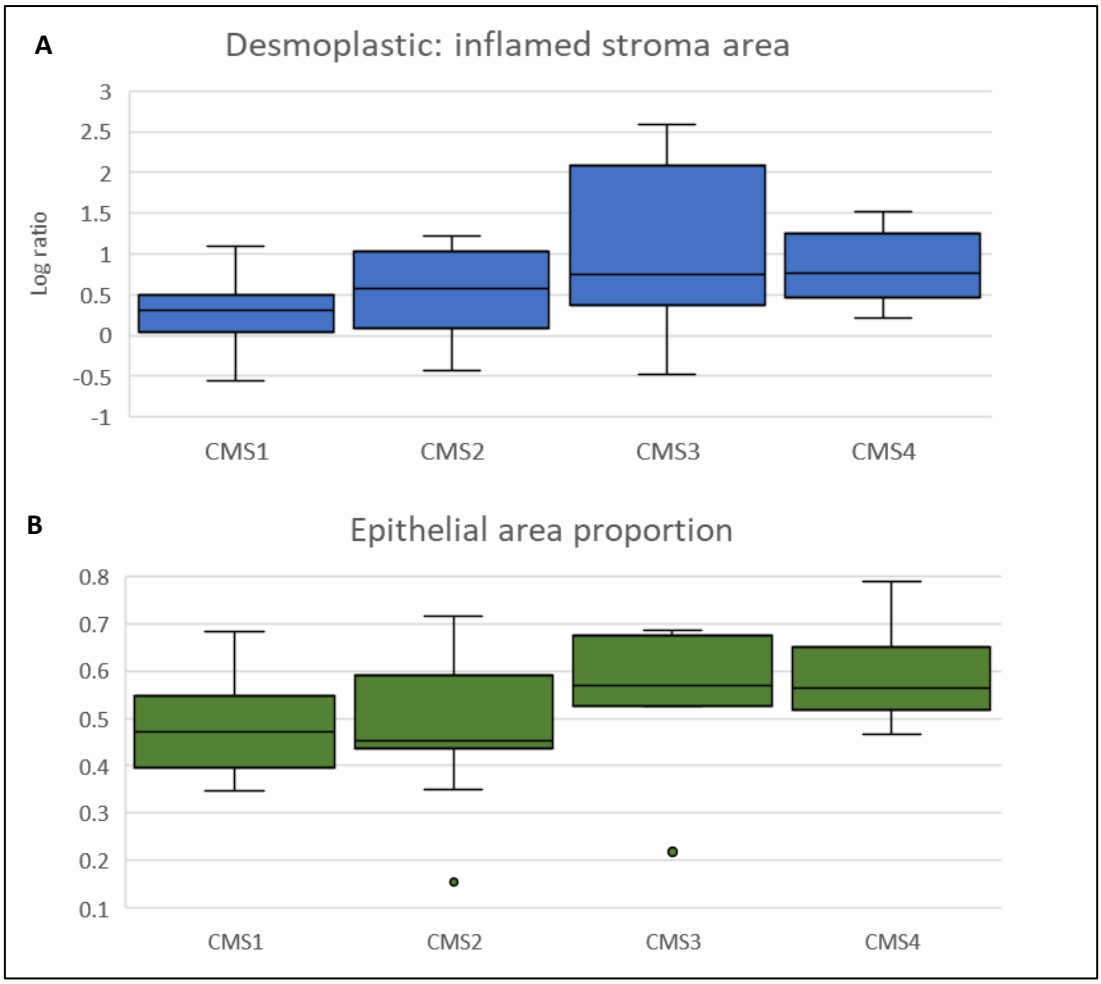


FIGURE 5.17: BOX AND WHISKER PLOTS SHOWING DIGITAL FEATURES IN 42 RECTAL CANCERS BY CMS GROUP. A. LOG RATIO OF DESMOPLASTIC TO INFLAMED STROMAL AREA, SHOWING NO SIGNIFICANT DIFFERENCE BETWEEN CMS GROUPS, P=0.096. **B.** VARYING EPITHELIAL PROPORTION OF TUMOUR BY CMS GROUP, P=0.078.

For 111 samples without prior radiotherapy, ESTIMATE[101] stroma and immune scores, epithelial signature score[103] and CMS classification were available. Figure 5.18 displays the range of scores for each CMS group. Kruskal-Wallis test indicates that the differences between groups were significant for each score: stroma score p<0.001; immune score p=0.016, epithelial score p<0.001.

The corresponding analysis was done for CRIS groups for 143 samples with available scores and there were no significant findings.

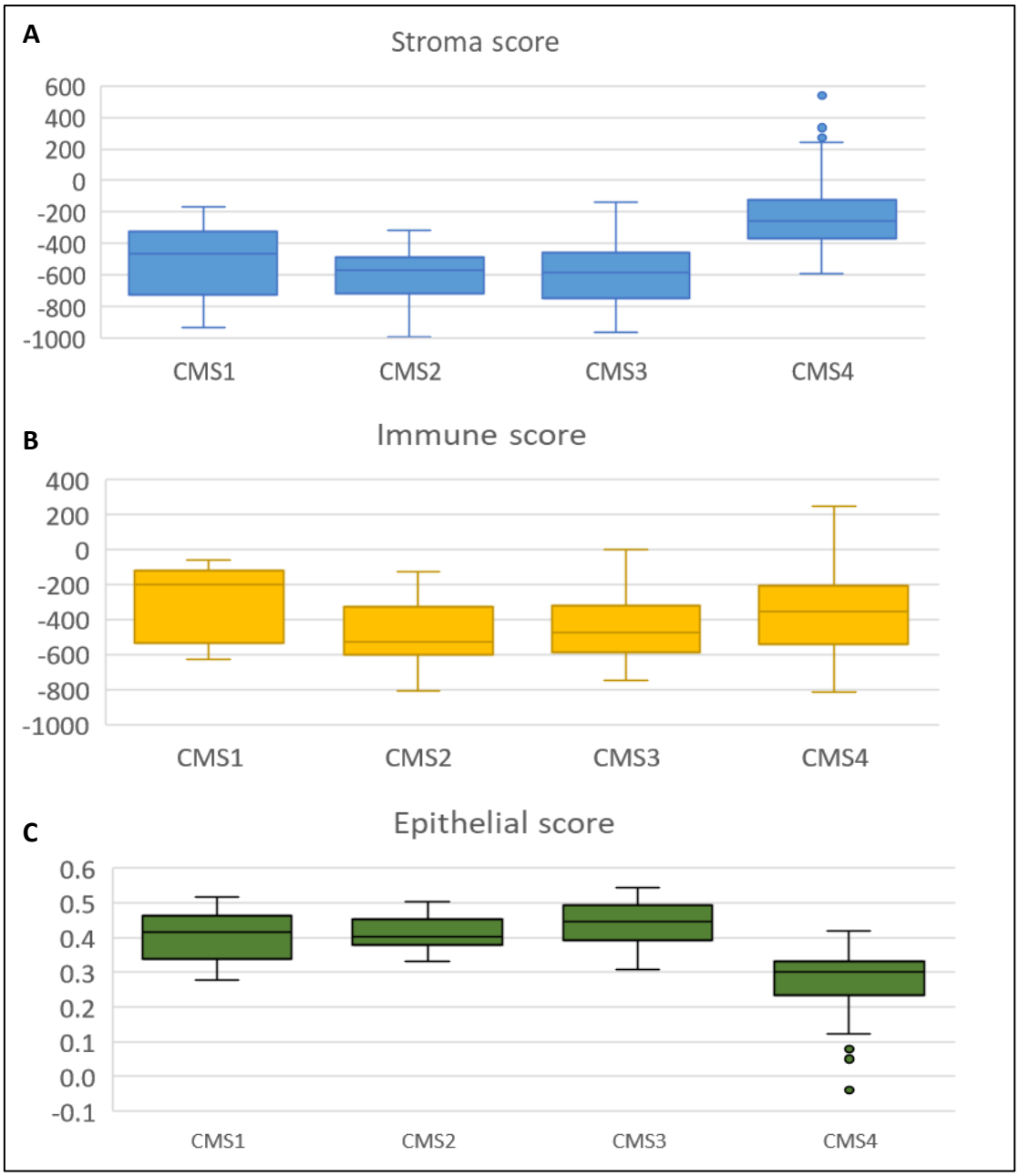


FIGURE 5.18: ESTIMATE SCORES AND EPITHELIAL SCORE FOR 111 RECTAL CANCERS BY CMS GROUP. A. ESTIMATE STROMA SCORE[101] FOR EACH CMS GROUP, KRUSKAL-WALLIS TEST GIVES P=1.1X10^{-10} INDICATING A HIGHLY SIGNIFICANT DIFFERENCE. **B.** ESTIMATE IMMUNE SCORE[101] FOR EACH CMS GROUP, KRUSKAL-WALLIS TEST GIVES P=0.016 INDICATING A SIGNIFICANT DIFFERENCE **C.** EPITHELIAL SCORE CALCULATED ACCORDING TO TAN *ET AL.*[103] FOR EACH CMS GROUP, KRUSKAL-WALLIS TEST GIVES P=5.1X 10^{-12} INDICATING A HIGHLY SIGNIFICANT DIFFERENCE.

SUMMARY OF MOLECULAR SUBTYPE FINDINGS

The smallest CMS group with 10% was CMS1, the MSI subtype, classically characterised by hypermutation, microsatellite instability and strong immune activation. This group included the

one patient who had HNPCC but did not include the tumour with loss of PSH2: this tumour was classified as CMS3. A feature of CMS1 is hypermutation and *BRAF* mutations. For those samples tested 63% showed a *KRAS* mutation but there were no *BRAF* mutations. Only six CMS1 tumours were tested on the 50 gene panel; two had no mutations, three had three and one had four mutations, involving *KRAS*, *TP53*, *APC* and one each of *PIK3CA*, *SMAD4*, *AKT1* and *FBXW7*. The CMS1 group had the highest Immune score and tended to have the lowest desmoplastic to inflamed stroma ratio. Another feature of CMS1 is worse survival after relapse. In this group, only two (12%) developed recurrence and both died of disease.

In the TEM dataset 17% were CMS2, less than the 37% in Guinney *et al*'s set. This group did not exhibit any strongly characteristic features.

CMS3 tumours made up 18%; this is the metabolic subtype, characterised by epithelial and metabolic dysregulation. Described features are mixed MSI status (the one MSI tumour was in this group) and *KRAS* mutations – this occurred in 78% where recorded.

CMS4 was the largest group in the TEM set, 27%. This is the mesenchymal subtype, characterised by TGF-β activation, stromal invasion, and angiogenesis. Consistent with this, this group had the highest stromal score and the lowest epithelial score. It also tended to have a higher proportion of desmoplastic to inflamed stroma. Recurrence occurred in 22% (10/46); of these 70% have died of disease.

The CRIS-A subgroup is characterised as mucinous and enriched in MSI or *KRAS* mutations. This CRIS-A group included one mucinous and no MSI tumours, but 67% of those tested (10/15) had a *KRAS* mutation. CRIS-E tumours are characterised by *TP53* mutations; in this population there was a *TP53* mutation in 5/6 who were tested.

Overall, the CMS classification provides a better fit to the present data than the CRIS system. Both these systems were developed for colorectal cancers in general, in series where rectal cancers and

early cancers are usually only a small minority. In contrast this TEM series comprises early rectal cancers, so some discrepancy is not unexpected. The genes considered in CRIS are specifically derived from the epithelial cancer cells rather than stromal cells. This may indicate that the stromal component in early rectal cancer has a relatively greater role in tumour behaviour than in the tumours used to develop the classification system. The association of desmoplastic to inflamed stroma ratio on DNN digital pathology with disease recurrence would support this.

5.3.1.5 Precursor polyp type

One hypothesis of this study is that rectal cancers that arise from traditional serrated adenoma (TSA) polyps, rather than the more common tubulo-villous adenomas (TVA), have a worse prognosis. This subsection assesses potential prognostic markers based on characteristic features of TSA polyps. Such polyps are known to feature *RSPO* fusions and *RNF-43* mutations which lead to ligand-dependent (LD) activation of the Wnt signalling pathway, rather than *APC* mutations common in TVAs which cause ligand-independent (LI) activation of the pathway. An alternate proposed method to detect LD tumours is low *AXIN2* expression. These features were assessed as potential prognostic markers in this set of rectal cancers.

FUSIONS

Of the 168 TEM samples only six showed a *PTPRK-RSPO3* fusion. No other fusions involving *RSPO2* or *3* were detected. Of these, three also showed high gene expression of *RSPO3* and epithelial *RSPO* expression on ISH staining. The other three showed low gene expression and no epithelial expression on *RSPO* on staining; the reason for this is unclear but it may reflect a defect in the fusion testing. The CMS was unclassified for two, CMS2 for one and CMS4 for three. Outcome data showed one of these six (17%), with high expression, died of disease, all others remained disease-free.

As *RSPO3* fusions are associated with high *RSPO3* expression this was also assessed. High expression was noted in 12 tumours, including three with fusions as above. Of the 12 there were two (17%) instances of recurrence; both of these patients died of disease. Figure 5.19A shows a dot plot of *RSPO3* expression and outcome.

In total 15 tumours had an *RSPO3* fusion and/or high *RSPO3* expression. Disease recurrence for this group was compared with the main group, considered to be LI tumours (Figure 5.20A). There was no significant difference between the groups (p=0.53).

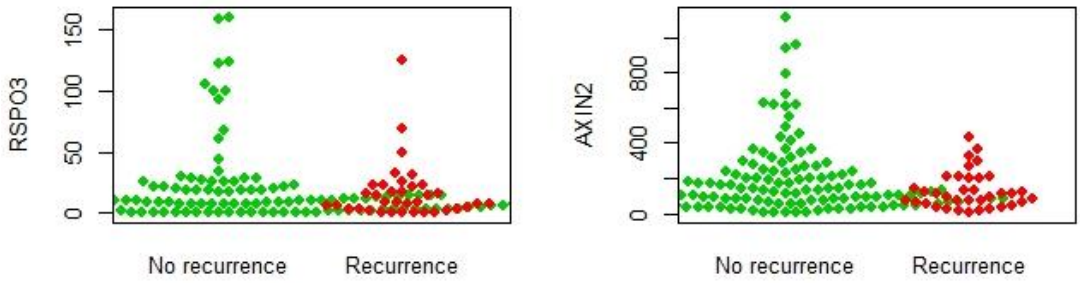


FIGURE 5.19: DOT PLOTS SHOWING GENE EXPRESSION ACCORDING TO DISEASE RECURRENCE. A. *RSPO3.* **B.** *AXIN2.*

AXIN2 EXPRESSION

A study of colorectal cancers which included some of these data concluded that low levels of *AXIN2* expression effectively discriminates between LI and LD tumours[171]. This was assessed further in the full set of 168 rectal cancers. Figure 5.19B shows a dot plot of *AXIN2* expression by outcome in this population.

Using the presence of fusion or high expression of *RSPO3* to define LI and LD groups, there was no significant difference in *AXIN2* levels between the groups (Mann-Whitney, p = 0.62). Taking the lowest decile of *AXIN2* expression to be low expression gave an alternate grouping of LD tumours. Only 3/17 of this group coincided with tumours defined as LD on the basis of *RSPO3* alterations, one had a fusion and two had high expression; none of these three had disease recurrence.

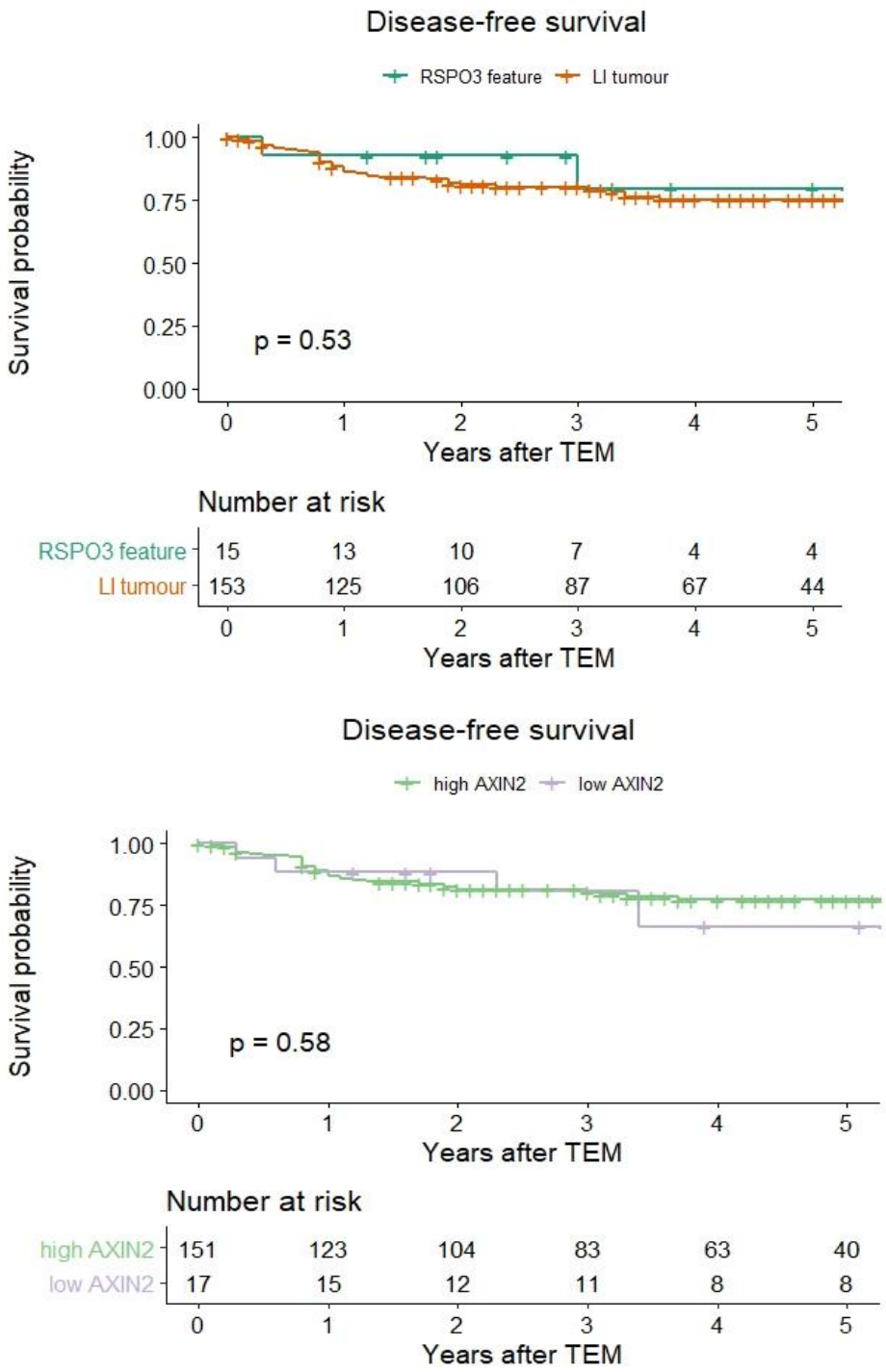


FIGURE 5.20: DISEASE-FREE SURVIVAL CURVES FOR PUTATIVE LD AND LI GROUPS OF RECTAL CANCER. A. LD GROUP DEFINED BY PRESENCE OF FUSION OR HIGH EXPRESSION OF *RSPO3*, COMPARED WITH LI GROUP WITHOUT RSPO3 ALTERATION. **B.** LD GROUP DEFINED BY LOWEST DECILE OF *AXIN2* EXPRESSION, COMPARED WITH LI GROUP WITH UPPER 90% *AXIN2* EXPRESSION.

Disease-free survival for the low-AXIN2 group was compared with the main group (Figure 5.20B), but again there was no significant difference between the groups (p=0.58).

In summary, neither *RSPO3* alterations nor low *AXIN2* expression has proven useful in identifying poor prognosis tumours in this group of early rectal cancers. This may be because neither method is accurately identifying LD tumours, or because LD, or TSA-origin, tumours do not have a different recurrence risk to TVA-origin cancers.

5.3.1.6 Differential gene expression

RECURRENCE VS NO RECURRENCE

Initially a binary analysis was undertaking comparing subgroups of patients who did and did not develop recurrence: 26 patients with poor prognosis were matched with 55 patients who were disease-free when censored, excluding any patients who had pre-operative radiotherapy. RNA sequencing data was compared looking for differentially expressed genes, accounting for sequencing batch. Five samples were considered outliers on quality control checking, two poor prognosis and three disease-free; these were excluded from the differential expression analysis, leaving 24 poor prognosis and 52 controls.

Differential expression analysis only detected one gene, *CASC15*, expressed at FDR 0.05 level, of a total 9,941 genes detected. A further 22 genes were differentially expressed at FDR 0.1 level (Figure 5.21A). Of these 23 genes, 14 were over-expressed in the poor prognosis group and nine were under-expressed.

18 of these genes were mapped to 29 significant Reactome pathways (FDR <0.05); five genes were not found on the Reactome system. In order to avoid over-analysis as the initial FDR restriction had been loosened, only pathways with more than one hit were considered further.

The immune system featured most prominently, with 12 of 29 pathways involving cytokine signalling and a further two involving innate immune system pathways. Figure 5.21B shows the significant pathways grouped and the main genes involved.

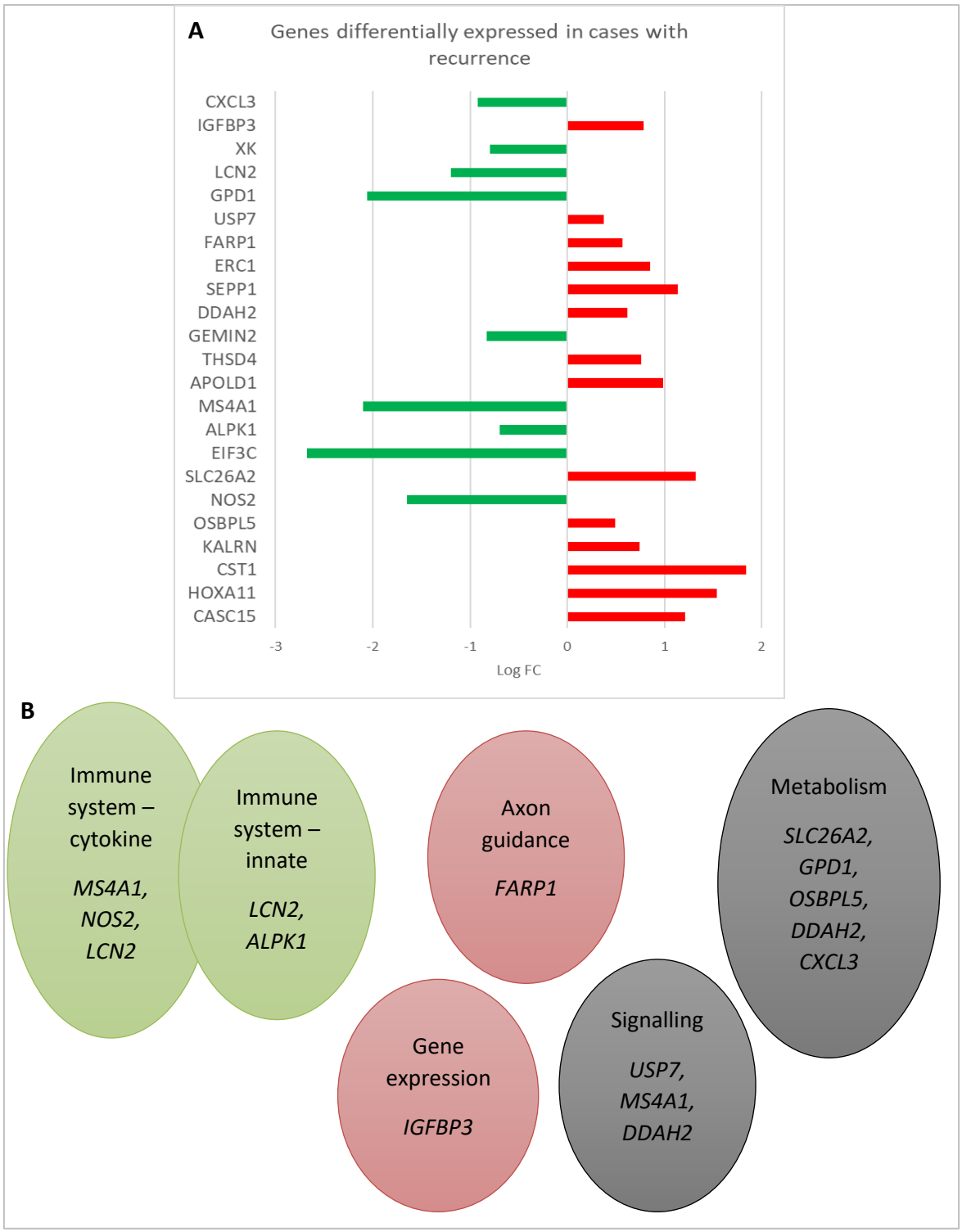


FIGURE 5.21: GENES DIFFERENTIALLY EXPRESSED IN THE POOR PROGNOSIS GROUP. A. DIFFERENTIAL EXPRESSION AT FDR <0.1 IN 23 GENES, INDICATING LOG FOLD CHANGE, RED= INCREASED EXPRESSION IN POOR PROGNOSIS TUMOURS, GREEN= REDUCED EXPRESSION IN POOR PROGNOSIS TUMOURS. **B.** DIAGRAM INDICATING REACTOME PATHWAYS THAT WERE SIGNIFICANTLY DIFFERENTIALLY EXPRESSED IN THE POOR PROGNOSIS TUMOURS. GREEN INDICATES ALL GENES HAD LOW EXPRESSION, RED INDICATES ALL GENES HAD INCREASED EXPRESSION, AND GREY INDICATES A MIX OF INCREASED AND REDUCED GENE EXPRESSION LEVELS IN THE PATHWAY.

SURVIVAL ANALYSIS

To further assess genes that may be significant in disease recurrence, Cox regression was used to assess the time till recurrence or censoring rather than the binary event of recurrence. Initially other potential factors: sequencing batch, tumour stage, pre-operative radiotherapy, post-TEM management pathway and CMS, were also considered in the total group of 168 patients. Only pre-operative radiotherapy had a significant effect, and in fact, the effect size of this on disease recurrence was so much larger than any other factor that subsequent analysis was restricted to the 150 patients who had not had pre-operative radiotherapy.

Cox regression univariate analysis was undertaken to assess genes that were individually significant in the time till disease recurrence, assessing the 50 genes with the lowest p values found in the binary analysis in the previous subsection. There were 17 significant genes with p value < 0.05, and a further 10 genes with p value between 0.05 and 0.10 which were retained at this stage; these genes are shown in the Table 5.1.

Of the 27 genes, 13 were positively associated with disease recurrence and 14 were negatively associated. The effect sizes were fairly small: the largest positive effect sizes were for *CASC15* and *HOXA11*; the largest negative effect sizes were for *MS4A1*, *NOS2* and *GEMIN2*. There was a substantial overlap of 15 genes compared with the binary analysis, including these five genes with largest effect sizes.

Time-dependent ROC (receiver operator curves) were calculated for each of the 27 genes to assess the individual gene's prediction performance for the risk of disease recurrence over the five years following TEM surgery. The AUCs (area under the curve) are also shown in Table 5.1. ROC and disease-free survival curves are shown in Figure 5.22 for the three genes with the highest AUC.

TABLE 5.1: GENES SIGNIFICANTLY ASSOCIATED WITH POORER SURVIVAL IN 150 RECTAL CANCERS WITHOUT PRIOR RADIOTHERAPY, ORDERED BY P VALUE AND ALSO SHOWING REGRESSION COEFFICIENT, HAZARD RATIO AND AUC FOR ROC DETERMINED BY KAPLAN-MEIER METHOD.

Gene	Coefficient	Hazard ratio (95% CI)	p value	AUC
CCDC77	0.0091	1.01 (1.01-1.01)	0.000014	0.584
ADIPOR2	0.0012	1 (1-1)	0.00010	0.527
CASC15	0.0135	1.01 (1.01-1.02)	0.00053	0.698
WNK1	0.0013	1 (1-1)	0.00076	0.547
ERC1	0.0043	1 (1-1.01)	0.0021	0.600
KRAS	0.0012	1 (1-1)	0.0027	0.559
KRT6B	0.0077	1.01 (1-1.01)	0.0041	0.534
CXCL1	-0.0033	0.997 (0.994-0.999)	0.0058	0.676
NOS2	-0.0327	0.968 (0.946-0.991)	0.0059	0.713
LCN2	-0.0015	0.999 (0.997-1)	0.0107	0.726
CXCL3	-0.0014	0.999 (0.997-1)	0.0154	0.645
MS4A1	-0.0581	0.944 (0.9-0.989)	0.0164	0.713
CASP1	-0.0061	0.994 (0.989-0.999)	0.0209	0.704
ALPK1	-0.0139	0.986 (0.973-0.999)	0.0398	0.638
LRG1	-0.0090	0.991 (0.982-1)	0.0432	0.655
SLC26A2	0.0007	1 (1-1)	0.0488	0.597
CXCL2	-0.0038	0.996 (0.993-1)	0.0496	0.595
SLC40A1	-0.0043	0.996 (0.992-1)	0.0503	0.600
APOLD1	0.0025	1 (1-1.01)	0.0546	0.568
XK	-0.0102	0.99 (0.979-1)	0.0576	0.640
UCA1	-0.0092	0.991 (0.982-1)	0.0579	0.609
IGFBP3	0.0014	1 (1-1)	0.0672	0.643
HOXA11	0.0102	1.01 (0.999-1.02)	0.0675	0.639
GEMIN2	-0.0195	0.981 (0.96-1)	0.0737	0.592
DDAH2	0.0008	1 (1-1)	0.0888	0.580
LINC.PINT	-0.0127	0.987 (0.973-1)	0.0914	0.603
CST1	0.0010	1 (1-1)	0.0968	0.655

The highest AUC obtained was 0.74 for *LCN2* (Figure 5.22A) and the optimal cut-point is the median *LCN2* expression value.

The three genes with the highest AUCs are all involved in cytokine signalling in the immune system. To assess the effect of low expression in this pathway, tumours were grouped into those with low expression of all three of these genes: *LCN2*, *NOS2* and *MS41A*, and all other tumours. This model defined a poor prognosis group comprising 18% (27/150) of the total.

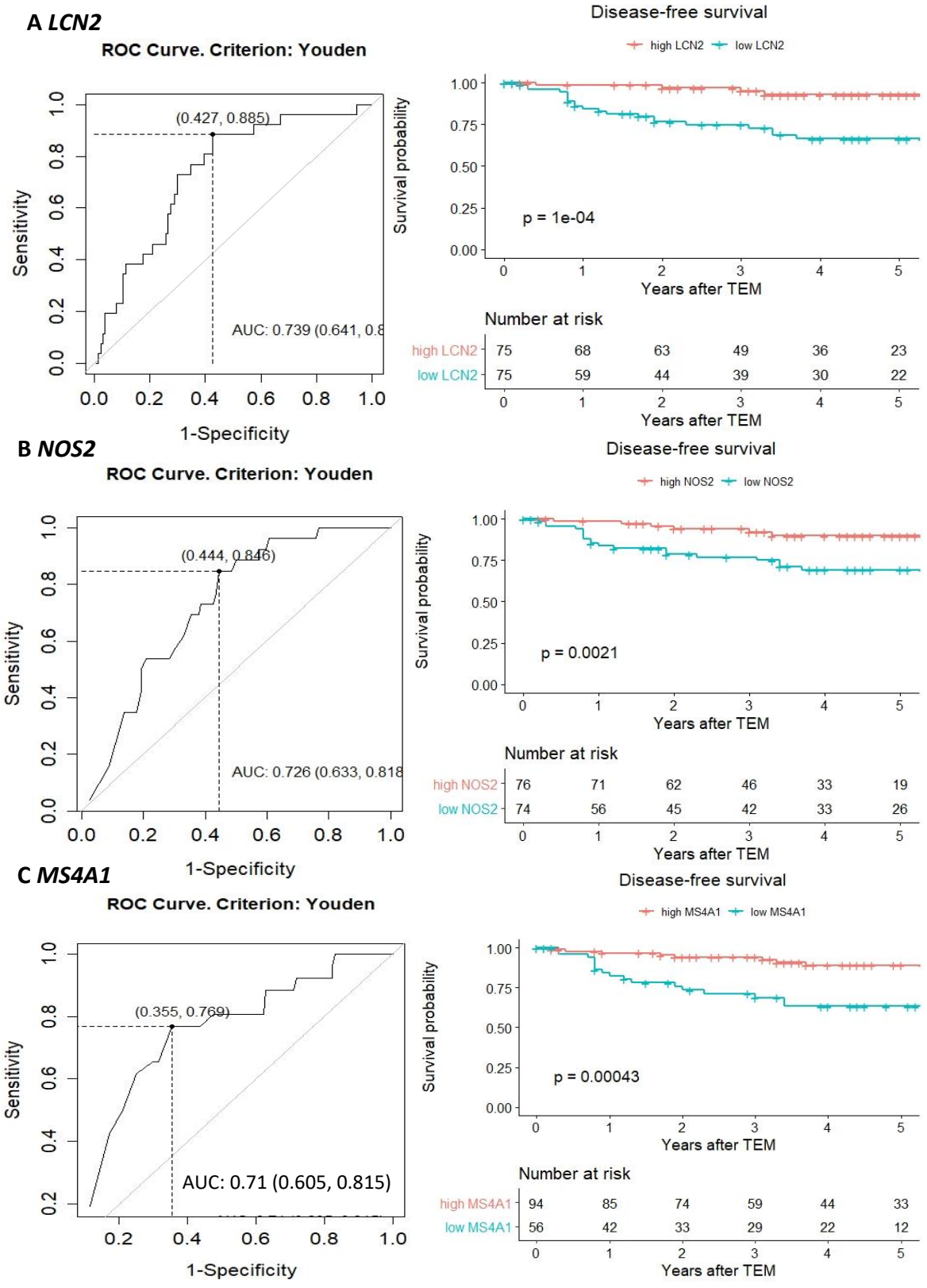


FIGURE 5.22: ROC AND DISEASE-FREE SURVIVAL CURVES FOR THE TOP THREE GENES, USING YOUDEN METHOD FOR ROC AND NEGATIVE OF GENE EXPRESSION. A. *LCN2* WITH AUC 0.74 AND A SIGNIFICANT DIFFERENCE IN DISEASE-FREE SURVIVAL, P=0.0001, **B.** *NOS2* WITH AUC 0.73 AND P=0.0021 FOR DIFFERENCE IN DISEASE-FREE SURVIVAL. **C.** *MS4A1* WITH AUC 0.71 AND P=0.0004 FOR DIFFERENCE IN DISEASE-FREE SURVIVAL.

The difference was highly significant (Figure 5.23) with 5-year disease-free survival of 39% (95% CI: 23-66) compared with 89% (95% CI: 82-95). This demonstrates that a reduced immune response in the tumour stroma is associated with a very poor prognosis.

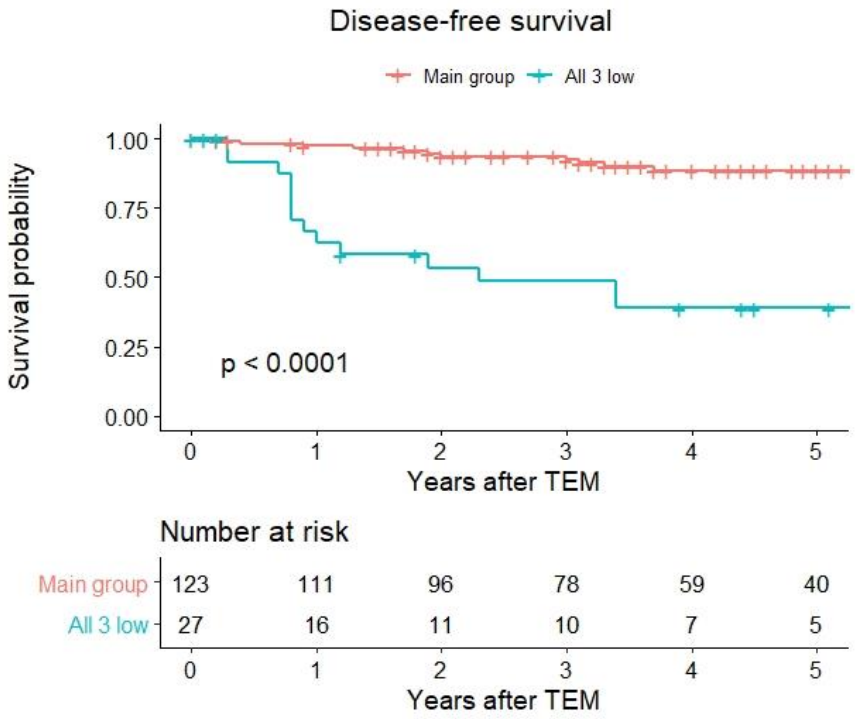


FIGURE 5.23: DISEASE-FREE SURVIVAL FOR GROUP WITH LOW EXPRESSION OF ALL THREE OF *LCN2*, *NOS2* AND *MS4A1*, COMPARED TO THE MAIN GROUP IN A TOTAL OF 150 RECTAL CANCERS.

Multivariable Cox regression analysis was then undertaken for disease recurrence. This model was based on 150 patients with 26 instances of disease recurrence. The 27 genes above were then fed into the multivariable Cox regression, and after successively removing the least significant genes the final model including five genes was obtained. This model had a concordance index of 0.846 and was highly significant on the likelihood ratio test ($p = 2 \times 10^{-9}$).

TABLE 5.2: FIVE-GENE MODEL FOR POOR PROGNOSIS

	Coefficient	Hazard ratio (95% CI)	P value
HOXA11	0.042	1.043 (1.022 - 1.065)	0.000070
TRPS1	0.023	1.024 (1.012 - 1.036)	0.000082
NOS2	-0.027	0.973 (0.951 - 0.996)	0.0236
ALPK1	-0.031	0.969 (0.950 - 0.989)	0.0026
MS4A1	-0.054	0.947 (0.905 - 0.992)	0.0205

Model diagnostics showed no evidence of non-proportional hazards (*HOXA11* p=0.31, *MS4A1* p= 0.94, *ALPK1* p=0.35, *TRPS1* p=0.06, *NOS2* p=0.62, global model p=0.29). Diagnostic plots are shown in Appendix 4.2. Schoenfeld residuals did not show any systematic trends over time. For each gene there are a small number of cases with high outlier results, but not the same case for each gene. After reviewing the outliers identified, it was decided to leave them in the model. The martingale residuals did not show any significant non-linearity in the model.

Using these five genes to create a linear predictor, the ROC gave AUC = 0.86. An optimal cut-point was determined and values with a high score had a significantly worse disease-free survival (Figure 5.24). This model defined a poor prognosis group comprising 46% (69/150) of the total. The 5-year disease-free survival was 56% (95% CI: 44-71) in those with a high score compared to 99% (95% CI: 96-100) in those with a low score.

An attempt was made to validate this five-gene signature on the TCGA dataset but there was insufficient outcome data on the 160-odd rectal cancer samples for meaningful interpretation.

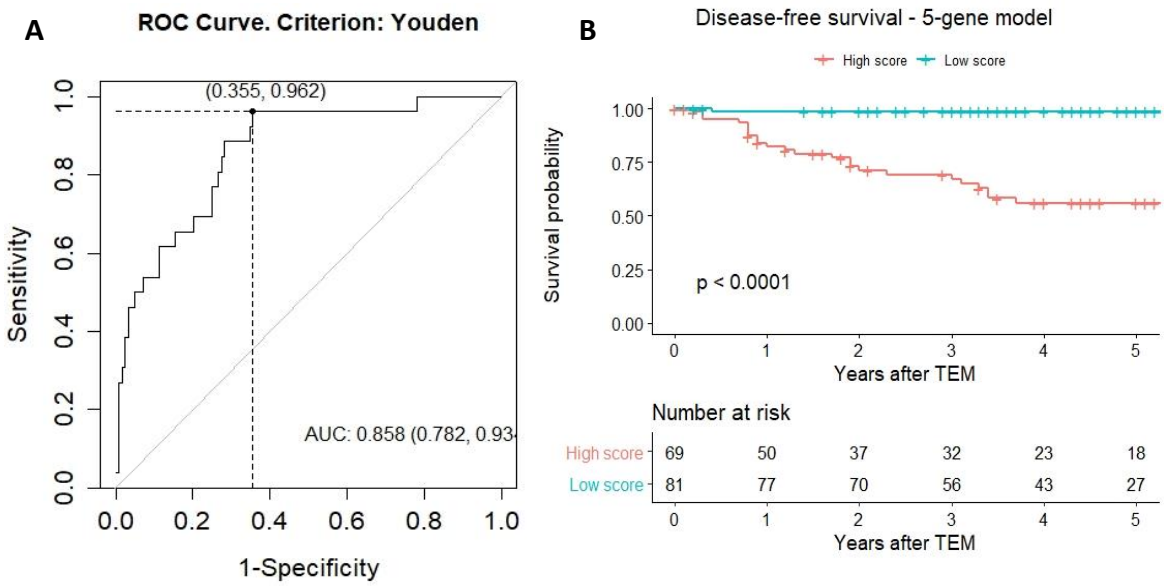


FIGURE 5.24: FIVE-GENE MODEL FOR DISEASE-FREE SURVIVAL IN RECTAL CANCER. A. ROC CURVE USING YOUDEN METHOD GAVE AUC=0.86. THE OPTIMAL CUT-POINT DETERMINED BY THIS MODEL WAS USED TO CALCULATE DISEASE-FREE SURVIVAL PROBABILITY FOR THE HIGH AND LOW SCORE GROUPS. **B.** DISEASE-FREE SURVIVAL CURVES FOR 150 RECTAL CANCERS SHOWING A SIGNIFICANT DIFFERENCE (P<0.0001) FOR GROUPS WITH HIGH AND LOW SCORES ON THE FIVE-GENE MODEL.

5.3.1.7 Summary of results for potential markers of poor prognosis

Table 5.3 summarises the findings in this section regarding potential markers of poor prognosis in the set of locally excised early rectal cancers.

TABLE 5.3: SUMMARY OF POTENTIAL PROGNOSTIC MARKERS IN EARLY RECTAL CANCER

Factor	Data requirement	Prognostic value
MSI status	Routine histopathology	Too uncommon in rectal cancer to be of value
Absence of *APC/ TP53/KRAS* mutation	50-gene mutation panel	Trend to shorter disease-free survival
Stromal fraction of tumour	H&E slide, marked and digital analysis	AUC: 0.58; 50% cut-off: 5-year DFS: 48% vs 77%, p=0.02; 38% cut-off: 5-yr DFS 63% vs 81%, p=0.004
Desmoplastic: inflamed stroma ratio	H&E slide, marked and DNN digital analysis	AUC: 0.71, significant difference in 5-year DFS: 53% vs 89%
ESTIMATE stromal and immune scores	Gene expression data: 141 genes for each	Not significant in this set
Chromatin heterogeneity	Nuclei extracted from tissue section and digital analysis	Not of value
Ploidy	Nuclei extracted from tissue section and digital analysis	Not of value
CMS classification	Gene expression data 693 genes	Not significant in this set
CRIS classification	Gene expression data 565 genes	Not of value
***RSPO* high +/or fusion**	Gene expression + targeted panel exome sequencing	Not of value
Low *AXIN2*	Gene expression data	Not of value
Low *LCN2*	Gene expression data	AUC: 0.74, highly significant difference in 5-year DFS: 93% vs 66%
Low *LCN2* & *NOS2* & *MS41A*	Gene expression data	Highly significant difference in 5-year DFS: 39% vs 89%
Five-gene model: *HOXA11, TRPS1, NOS2, ALPK1, MS4A1*	Gene expression data	AUC: 0.86, highly significant difference in 5-year DFS: 56% vs 99%

5.3.2 Radiosensitivity: outcome after adjuvant radiotherapy

5.3.1.1 Recurrence after adjuvant radiotherapy

There were 37 patients who received adjuvant radiotherapy; these were classified into two groups: seven developed recurrence after radiotherapy while 30 remained disease-free at last follow-up. There were five outliers, all in the disease-free group who were excluded from the differential expression analysis.

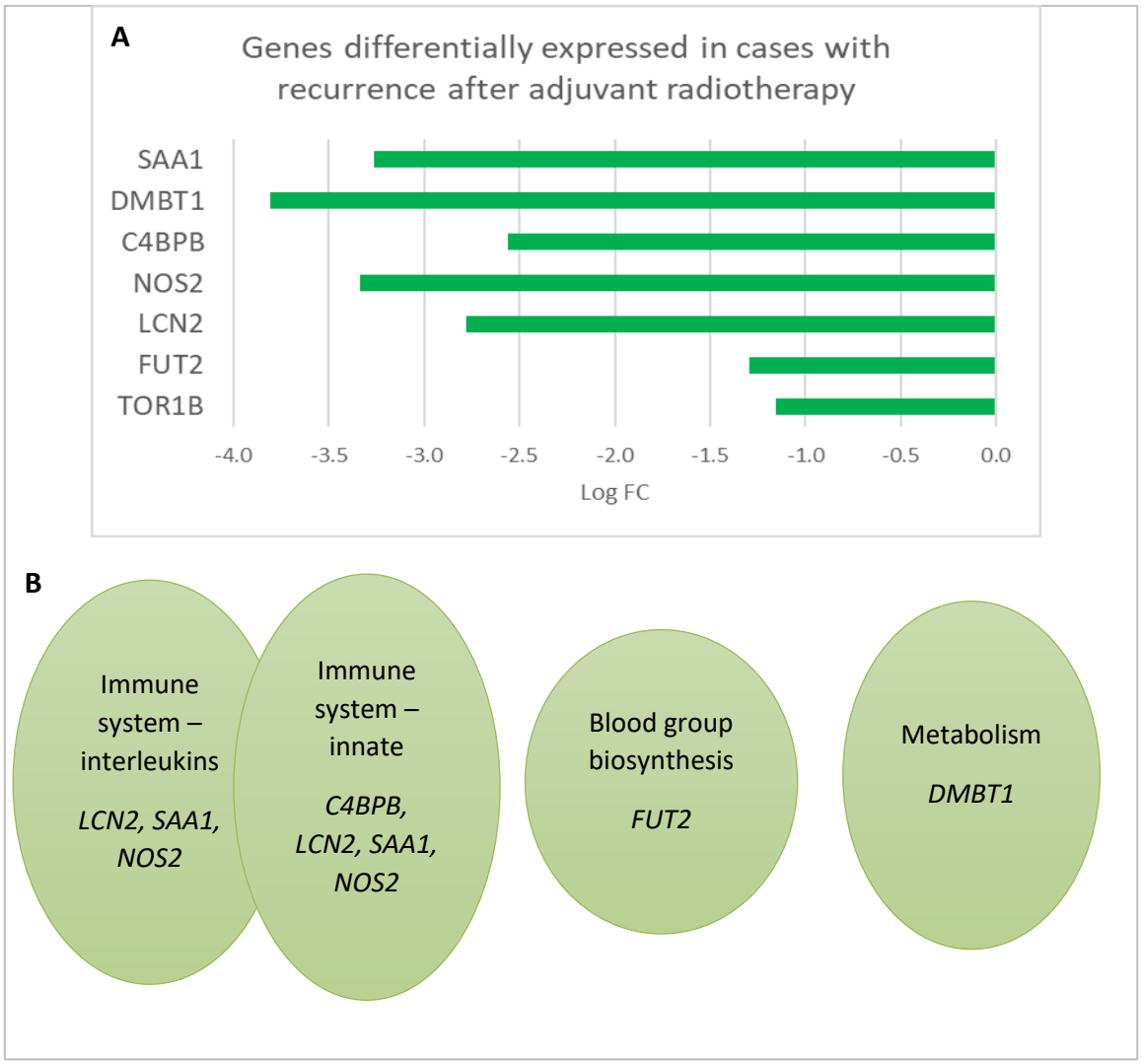


FIGURE 5.25: GENES DIFFERENTIALLY EXPRESSED IN THE GROUP WITH RECURRENCE AFTER ADJUVANT RADIOTHERAPY. A. DIFFERENTIAL EXPRESSION AT FDR <0.1 IN 7 GENES, INDICATING LOG FOLD CHANGE, GREEN= REDUCED EXPRESSION IN GROUP THAT DEVELOPED RECURRENCE AFTER ADJUVANT RADIOTHERAPY. **B.** DIAGRAM INDICATING REACTOME PATHWAYS THAT WERE SIGNIFICANTLY DIFFERENTIALLY EXPRESSED IN THE RECURRENT AFTER ADJUVANT RADIOTHERAPY TUMOURS. GREEN INDICATES ALL GENES HAD LOW EXPRESSION LEVELS IN THE PATHWAY.

Differential expression analysis only detected four genes at FDR 0.05 level of a total 10,610 genes: *C4BPB*, *DMBT1*, *NOS2* and *SAA1*. A further 3 genes were differentially expressed at FDR 0.1 level: *FUT2*, *LCN2* and *TOR1B*. All these seven genes were under-expressed in the group that developed recurrence following adjuvant radiotherapy (Figure 5.25). Two of these were also under-expressed in the poor prognosis group discussed above: *NOS2* and *LCN2*. These genes appeared in 63 Reactome pathways, 17 at FDR <0.05. Table 5.4 lists the significant pathways, grouped by relevant mapped proteins.

TABLE 5.4: REACTOME PATHWAYS MOST SIGNIFICANTLY DIFFERENTIALLY EXPRESSED IN GROUP WITH RECURRENCE AFTER ADJUVANT RADIOTHERAPY

Pathway name	Mapped entities
Interleukin-4 and Interleukin-13 signalling	*LCN2; SAA1; NOS2*
Signalling by Interleukins	*LCN2; SAA1; NOS2*
Cytokine Signalling in Immune system	*LCN2; SAA1; NOS2*
Immune System	*C4BPB; LCN2; SAA1; NOS2*
Metal sequestration by antimicrobial proteins	*LCN2*
Formyl peptide receptors bind formyl peptides and other ligands	*SAA1*
Advanced glycosylation end product receptor signalling	*SAA1*
Scavenging by Class B Receptors	*SAA1*
TRAF6 mediated NF-κB activation	*SAA1*
TAK1 activates NF-κB by phosphorylation and activation of IKKs complex	*SAA1*
Nitric oxide stimulates guanylate cyclase	*NOS2*
Peroxisomal protein import	*NOS2*
ABO blood group biosynthesis	*FUT2*
Lewis blood group biosynthesis	*FUT2*
Blood group systems biosynthesis	*FUT2*
Surfactant metabolism	*DMBT1*

The most significant pathways are concerned with the immune system, in particular cytokine signalling by interleukins. Part of the innate immune system includes antimicrobial proteins among whose actions is excluding essential metals from microbes and so restricting microbial growth. Another major inflammatory pathway involves NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) which is stimulated by activation of receptors for advanced glycosylation end products (RAGE).

5.3.1.2 Survival analysis

A further three patients were identified who had adjuvant radiotherapy, so the subsequent analysis included 40 patients with 8 instances of disease recurrence. A plot of disease-free survival for the whole group is shown in Figure 5.26A.

Cox regression was undertaken to assess genes that were individually significant in survival till time of disease recurrence or censoring rather than the binary event of recurrence. Other potential factors were initially considered: sequencing batch, tumour stage and CMS, but only positive resection margin was found to have a significant effect. The 22 genes with the lowest p values on the binary analysis of recurrence were assessed. There were six significant genes with a $p < 0.05$, and a further one gene with a p value between 0.05-0.10 which was retained at this stage. These are shown in Table 5.5.

TABLE 5.5: GENES SIGNIFICANTLY ASSOCIATED WITH RECURRENCE AFTER ADJUVANT RADIOTHERAPY IN 37 LOCALLY EXCISED RECTAL CANCERS, ORDERED BY P VALUE AND ALSO SHOWING REGRESSION COEFFICIENT, HAZARD RATIO, P VALUE AND AUC FOR ROC DETERMINED BY KAPLAN-MEIER METHOD WHERE P<0.05.

Gene	Coefficient	Hazard ratio (95% CI)	p value	AUC
NUPR1	0.0012	1.00 (1.00-1.00)	0.0062	0.810
CBX6	0.006	1.01 (1.00-1.01)	0.002	0.811
GADD45A	0.026	1.03 (1.01-1.05)	0.012	0.805
COL8A2	0.028	1.03 (1.00-1.05)	0.022	0.770 2 cut-offs
PRKCI	0.019	1.02 (1.00-1.04)	0.034	0.698
IRF2BPL	0.016	1.02 (1.00-1.03)	0.037	0.723
CTSF	0.020	1.02 (1.00-1.04)	0.052	0.701
Positive resection margin	1.65	5.2 (1.04-26.0)	0.045	n/a

In contrast to the differentially expressed genes detected on binary analysis which were all expressed at a lower level in the group with recurrence, all significant genes in the survival analysis were more highly expressed in those who developed recurrence. Time-dependent ROCs were plotted for the significant genes to assess the individual gene's prediction performance for the risk of disease recurrence after adjuvant radiotherapy. The AUCs are shown in Table 5.5. The highest AUCs obtained were 0.811 for *CBX6* and 0.810 for *NUPR1* (Figure 5.26B&C).

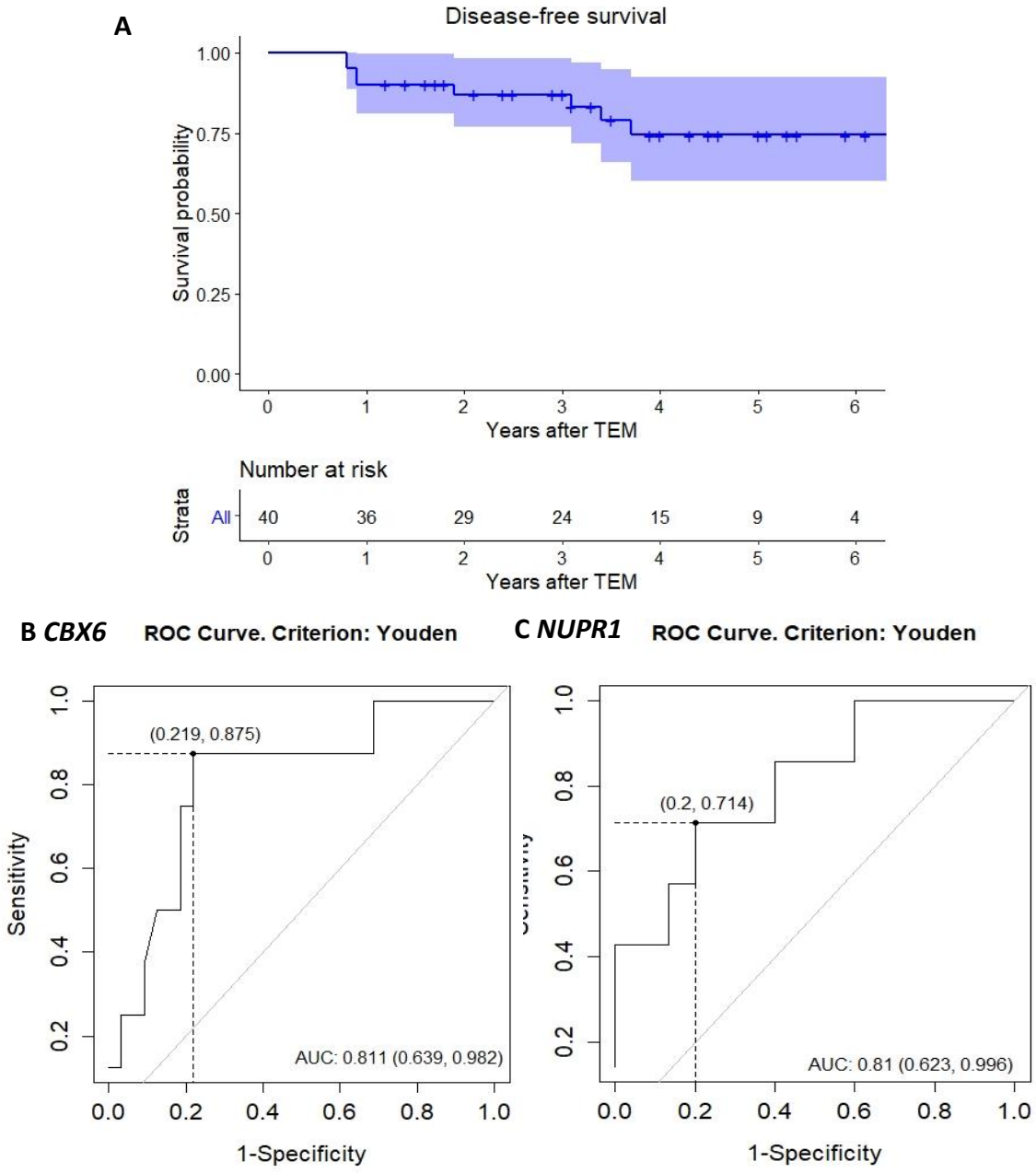


FIGURE 5.26: ADJUVANT RADIOTHERAPY AFTER LOCAL EXCISION OF RECTAL CANCER. A. DISEASE-FREE SURVIVAL FOR 40 PATIENTS**. B.** ROC CURVE FOR CBX6 WITH AUC 0.811. **C.** ROC CURVE FOR NUPR1 WITH AUC 0.810.

Multivariable Cox regression analysis was undertaken for disease recurrence. This model was based on 40 patients with 8 instances of disease recurrence. The genes retained from the univariate analysis were assessed. Resection margin was also included although this became non-significant and was subsequently removed from the model. After successively removing the least significant

variable the final model included two genes, *NUPR1* and *COL8A2*. This model had a concordance index of 0.821 and was significant on the likelihood ratio test (p = 0.004).

TABLE 5.6: TWO-GENE MODEL FOR RECURRENCE AFTER ADJUVANT RADIOTHERAPY.

	Coefficient	Hazard ratio (95% CI)	p value
NUPR1	0.0016	1.002 (1.001 - 1.003)	0.0028
COL8A2	0.037	1.038 (1.009 – 1.067)	0.010

Model diagnostics showed no evidence of non-proportional hazards (*NUPR1* p=0.38, *COL8A2* p= 0.12, global model p=0.09). Diagnostic plots are shown in Appendix 4.3. Schoenfeld residuals did not show any systematic trends over time. Index plots for influential cases showed a few outliers, but no observation was individually highly influential. Martingale residuals did not show any significant non-linearity.

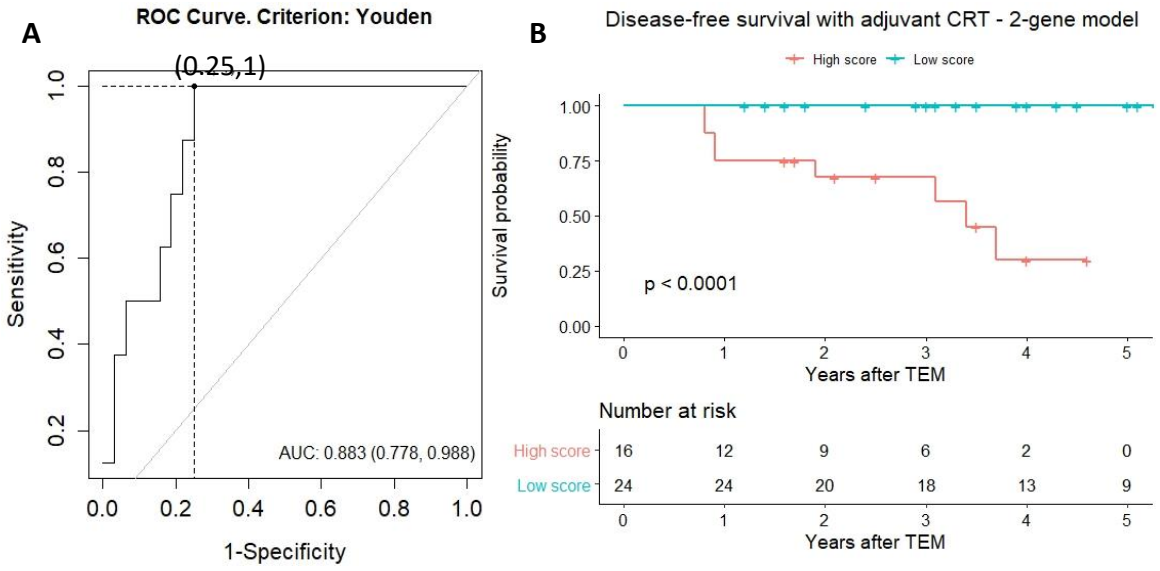


FIGURE 5.27: 2-GENE MODEL FOR DISEASE-FREE SURVIVAL AFTER ADJUVANT RADIOTHERAPY IN RECTAL CANCER. A. ROC CURVE USING YOUDEN METHOD GAVE AUC=0.88. THE OPTIMAL CUT-POINT DETERMINED BY THIS MODEL WAS USED TO CALCULATE DISEASE-FREE SURVIVAL PROBABILITY FOR THE HIGH AND LOW SCORE GROUPS. **B.** DISEASE-FREE SURVIVAL CURVES FOR 40 RECTAL CANCERS MANAGED WITH LOCAL EXCISION THEN ADJUVANT RADIOTHERAPY, SHOWING A SIGNIFICANT DIFFERENCE (P<0.0001) FOR GROUPS WITH HIGH AND LOW SCORES ON THE TWO-GENE MODEL.

Using this two-gene model to create a linear predictor, the ROC curve gave AUC = 0.88. An optimal cut-point was determined and values with a high score had a significantly worse disease-free

survival (Figure 5.27). The three-year disease-free survival was 68% (95% CI: 48-96) in those with a high score compared to 100% (95% CI: 100-100) in those with a low score.

The small number of patients and recurrences limit confidence in the model. It has not yet been possible to find another suitably large group of patients having adjuvant radiotherapy after local excision by TEM with adequate follow-up data to be able to validate this model.

None of the seven genes that demonstrated differential expression in the binary analysis of those who did and did not develop recurrence were significantly associated with survival when assessed individually in a Cox regression model. Nonetheless, to investigate whether a model using these genes might have a role, potentially with a larger dataset, the two of these seven genes with the highest AUCs, *C4BPB* and *LCN2*, were combined into a variable for low *C4BPB* and low *LCN2* or otherwise. Figure 5.28 shows that those with both low scores tended to have more recurrence, but the difference was not significant (p = 0.19).

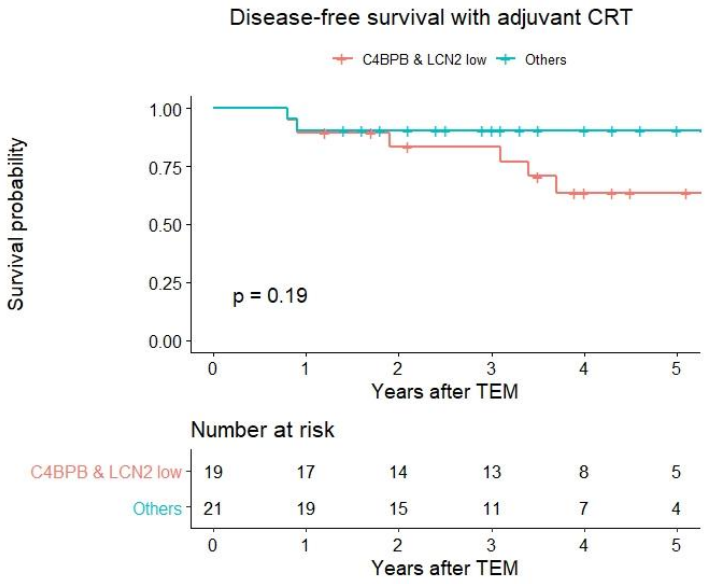


FIGURE 5.28: DISEASE-FREE SURVIVAL CURVES WITH ADJUVANT RADIOTHERAPY AFTER TEM IN GROUP WITH LOW *C4BPB* AND LOW *LCN2* COMPARED TO OTHERS IN 40 RECTAL CANCERS.

5.4 Discussion

This chapter has assessed various potential markers for predicting prognosis in the rectal cancer tissue of patients undergoing local excision of early rectal cancer. These have covered additional histopathology variables, digital pathology and molecular analysis.

MSI status is a core data item for locally excised rectal cancers. In this population, MSI tumours were extremely uncommon. This is an expected finding in rectal cancer but means it has little prognostic value. The 50-gene mutation panel now routinely undertaken as part of the histopathological assessment of these tumours showed a low level of mutations, as expected in rectal cancers. The high frequency of *APC*, *TP53* and *KRAS* mutations seen in this series is consistent with the series reported by Smith *et al.*[172]. Their study discusses colorectal tumours in general, but they include 41 rectal cancers and found similar levels of these mutations, between 39-51% for each. The worse prognosis for those without at least one of these three common mutations suggests that there is a less common pathway, not detected by one of the 50 common gene mutations, giving rise to rectal cancers with an intrinsically worse prognosis; this pathway may arise in a different precursor polyp type, for example the serrated pathway.

The proportion of stroma in a tumour, here reported as stromal fraction and often referred to as TSR in the literature, is not currently a core data item although with digitised methods making measurements more reliable it is being gaining prominence and may soon be incorporated into the standard TNM classification[54]. The standard cut-off used is 50% but in our series a cut-off of 38% provided better discrimination between those who did and did not develop recurrence, as only 9% of tumours had ≥50% stroma. This is lower than other reported studies, for example 34% of 377 rectal cancer in the QUASAR trial[55] which may be because the current series comprises early rectal cancers, which may not yet have produced a great volume of stroma. Nevertheless, even with a 38% cut-off the AUC was only 0.58 and the 5-year estimated disease-free survival for the two groups: 63% vs 81%, indicates that discrimination is not particularly good. The predictive value is

less than some reported series which again may reflect that these cancers are early and more subtle features of the stroma may have a greater role than simply the volume. The combination with ploidy status was more prognostic, indicating an accumulation of damage within the epithelial cancer and stroma has a cumulative effect on prognosis.

The ESTIMATE stromal score[101] infers the fraction of stromal cells in the tumour from expression of a 141-gene signature. For this population, the correlation between stromal fraction and stromal score had R^2 of only 0.17 and the stromal score did not have prognostic value. Similarly, the ESTIMATE immune score, designed to estimate the proportion of immune cells in the tumour, was not prognostic.

On the other hand, the ratio of desmoplastic to inflamed stroma measured by DNN digital pathology provided very good discrimination between good and poor prognosis tumours with 5-year estimated disease-free survival of 53% where the ratio is over 4.7 and 89% otherwise. Desmoplastic stroma is rich in CAFs and has no immune cell infiltration. High desmoplastic stromal content is often associated with TGF-β signalling activation and poor prognosis. In contrast, inflamed stroma is rich in lymphocytes. It is generally associated with immune activation and tends to be associated with a favourable outcome when it predominates. There is no data in the literature yet on artificial intelligence derived classification of histomorphology in this setting and recurrence risk, so this significant finding is very promising. One advantage of this as a prognostic marker is that is requires only an H&E stained slide, which is obtained in the routine processing of every tumour sample, a digital scanner, which is now becoming commonplace, and access to the DNN algorithm to analyse the slide, so results could be obtained rapidly in contrast to molecular methods where results may take months.

The superior prognostic value of the DNN derived method compared with the ESTIMATE scores is an interesting finding. The ESTIMATE method uses gene expression to determine the stromal and immune components and defines tumour purity as the remainder. It does not take account of any

other normal content such as vascular and muscle cells, and considers these as tumour cells, whereas DNN segmentation includes mesenchyme. The tumour purity is therefore overestimated if there is a significant amount of mesenchyme and this may explain the low correlation found in this dataset between tumour purity and epithelial area proportion measured by DNN ($R^2 = 0.27$). It is also possible that inflammatory genes expressed by tumour cells, as opposed to immune cells in the stroma, can cause the ESTIMATE immune score to be over-estimated. This could explain the poor correlation between the immune score and the DNN inflamed stroma area ($R^2 = 0.30$).

The DNN segmentation also showed significant differences in cellular density in the tumour compartments with high density in the inflamed stroma with an immune lymphocyte-rich infiltrate, low density in the desmoplastic stroma and the tumour epithelial areas having an intermediate density. The differences between proportional area and proportional cell count may explain differing results when looking at different methods for determining tumour vs non-tumour or stromal proportion.

Overall, the results obtained with DNN, providing artificial intelligence-based tissue segmentation, are more consistent and have greater prognostic value than other methods used, even though the DNN results were available for only about a third of cases.

The CMS classification appeared better suited to this population of early rectal cancers than the CRIS. Both the DNN segmentation of the tumour and the molecular scores showed findings consistent with the CMS subtype descriptions proportion. The CMS classification however did not have prognostic value in this population.

The other digital techniques employed in this chapter, nucleotyping and ploidy, surprisingly failed to show any value in predicting disease recurrence. Both have been shown useful in colorectal cancer[51,64]. The only obvious difference is that the present population is a relatively homogeneous group of early rectal cancers as opposed to the wide variety of stages of colorectal cancers generally studied. This implies that the nuclear and DNA features that have been shown significant elsewhere

are not predictive in the early stages of cancer growth but become significant as the cancer progresses.

Another potential prognostic factor assessed in this chapter was the histogenic origin of the cancer. The hypothesis that cancers derived from TSA polyps have a worse prognosis could not be confirmed. Molecular features of ligand-dependent activation of the Wnt pathway include *RSPO3* fusion or high expression and low *AXIN2* expression. Neither of these had prognostic value in this dataset. Only six (3.6%) cancers showed an *RSPO3* fusion and another nine (5.4%) showed high *RSPO3* expression in the absence of fusion. The majority of the fusion and/or high-expression tumours were CMS4, the subtype associated with more stromal infiltration and worse prognosis. For this group, the recurrence rate was 17% but numbers are too small for any definitive conclusion. Another feature is *RNF43* mutations, and although samples were sent for processing, results were not available. The failure to find an association between TSA-origin cancers and prognosis may either be due to the absence of an association or inadequate identification of cancers arising from TSA polyps. McCarthy *et al*'s recent review of TSA[173] considered a variety of other molecular markers but did not find any others of great value.

5.4.1 Molecular model for disease recurrence

As a single gene marker, low expression of *LCN2* provided good prognostic value with 5-year disease-free survival of 66% vs 93% for high expression; half the patients were in each group. *LCN2* (also known as *NGAL*) is a secreted acute phase protein and is upregulated in epithelial cells in inflammatory conditions[174]. It is bactericidal, by sequestering iron and so limiting bacterial growth[175]. Its role in cancer is attributed to its function in the innate immune system[176]. Low expression has been associated with high levels of the TNF receptor (DR5, also known as TNFRSF10B) which mediates apoptosis[177].

When combined with low expressions of *NOS2* and *MS41A* a much smaller high-risk group of 18% was identified. This group had with 5-year disease-free survival of 39% vs 89% for the majority. This indicates that low expression of the various components of the innate immune system is additive in effect on recurrence risk. Although effectively identifying an extremely poor prognosis group, the collection of others into a single group is less informative. The impact of the immune status on prognosis is consistent with Galon *et al*'s work on the Immunoscore[178].

NOS2 (nitric oxide synthase 2, inducible) is an enzyme that catalyses production of nitric oxide and is mainly expressed by immune cells. Its levels are reduced by *TP53*[179]. It is a marker of macrophage activation in colorectal cancer[180]. Activated macrophages also produce high levels of cytokines that promote inflammation and T helper cell activity. One of the other differentially expressed genes that does not form part of this model but shows higher expression in poor prognosis tumours is *DDAH2* which inhibits the generation of nitric oxide. The combination of low *NOS2* and high *DDAH2* suggests that nitric oxide is playing an important, protective role in this population of early rectal cancers. Keshet and Erez[181] note that NO is involved in the initiation and progression of cancer, but also contributes to the immune response against the tumour and limits proliferation and invasion of the cancer. According to Vahora *et al.*[179] high levels of NO cause DNA damage and apoptosis while medium levels promote metastasis and inhibit apoptosis and low levels promote angiogenesis and tumour proliferation. NO is known to promote radiosensitivity in tumours. Cook *et al.*[182] demonstrated that NO acts synergistically with ionising radiation in colorectal cancer cells to promote apoptosis and inhibit tumour growth by activating *TP53*. Of the other upregulated genes in the poor prognosis group *IGFBP3* and *USP7* also regulate *TP53*.

MS4A1 encodes CD20, a B lymphocyte antigen which contributes to the development of B cells and their differentiation into plasma cells. CD20 allows a strong B-cell immune response. It is a marker for B cells, specifically tumour-infiltrating B lymphocytes (TIL-B) and plasma cells[183]. Bindea *et al.*[184] found high expression of *MS4A1* and another B-cell marker, CD19, correlated with longer disease-

free survival. They also found that B cells are strongly correlated with T helper cells in colorectal cancers and a relative lack of B cells indicates a reduced immune response in a tumour.

5.4.1.1 Five-gene model for poor prognosis

The five-gene model for disease-free survival in patients that had not had prior radiotherapy included increased *HOXA11* and *TRPS1* and decreased *MS4A1*, *ALPK1* and *NOS2*. The model classified 46% of patients as high risk, with 5-year disease-free survival of 56% compared to 99% for the low-risk majority.

HOXA11 (homeobox A11) is part of a cluster of genes that encode transcription factors and control many processes including cell differentiation, motility, and apoptosis. Abnormal expression of *HOXA11* has been noted in glioblastoma[185], gastric[186], and pancreatic cancer[187] where low expression correlates with poorer prognosis. Rodrigues *et al.*[186] note that the effects of HOX genes are tissue-specific, and both up and down-regulation can have tumorigenic effects on growth and differentiation.

TRPS1 also encodes transcription factors and represses GATA-regulated genes. It has effects on cell proliferation and the control of cell cycle progression[188] and has been shown to be increased in breast cancer[189,190]. Hong *et al.*[191] found that *TRPS1* expression was increased in colon cancer tissue compared with surrounding normal tissue, and higher expression correlated with higher stage and lymph node metastases.

So, both the more highly expressed genes in the recurrence model encode transcription factors while the three less expressed genes are involved in the immune system. Two, *LCN2* and *MS4A1*, have been discussed above. *ALPK1* is a pattern recognition receptor involved in activating NF-κB signalling which promotes an inflammatory response[192]. A sugar, HBP, produced by Gram-negative bacteria activates *ALPK1*, leading to neutrophil recruitment and the production of several cytokines

and chemokines. This suggests *ALPK1* normally forms part of the body's defence against gut organisms, and it has been implicated in colitis and colitis-associated colorectal cancer[193].

In summary, the model indicates increased encoding of transcription factors and reduced immune response are features of tumours that are likely to recur.

5.4.2 Molecular model for resistance to adjuvant radiotherapy

One quarter of the patients whose tissue samples were analysed in this chapter underwent adjuvant radiotherapy after local excision of the cancer because of histopathological high-risk features. Although radiotherapy reduced the expected incidence of recurrence, recurrence does still occur, and these patients have been exposed to radiotherapy without the benefit of subsequent non-recurrence of the cancer. The patients analysed in this section had all the macroscopic tumour removed by TEM, so only microscopic disease in the bowel wall, mesorectum or possibly as distant micrometastases could remain. Gene expression data comparing those who subsequently developed recurrence and those who did not identified seven differentially expressed genes, all of which were downregulated in the recurrence group; the four most significant are *C4BPB*, *DMBT1*, *NOS2* and *SAA1*. As with the poor prognosis models discussed above, most of these genes are involved in the immune system or inflammatory process. Two of the genes have been associated with radioresistance: *NOS2*[194] and *C4BPB*[195].

C4BPB (complement 4 binding protein, β chain) has an important role in the innate immune response. There is little specific information about *C4BPB* in rectal cancer. Reis *et al.*'s review[196] notes that imbalanced complement activation in the tumour microenvironment is linked to inflammation and suppression of anti-tumour immune responses. Specifically, C4BP binds to apoptotic and necrotic cells and amyloid protein to prevent inflammation. Li *et al.*[197] found *C4BPB* to be one of the top 10 significantly under-expressed genes in rectal cancer compared to colon cancer, while Yokoi *et al.*[195] found it to be one of 26 'radiation-resistance' genes in CRC cell lines.

SAA1 (serum amyloid A) is an acute phase protein with an important role in the inflammatory process. A systematic review[198] notes that high levels have been reported in many cancers including colorectal and increasing concentrations correlate with severity of cancer stage. A review of *SAA1* in tumour pathogenesis[199] noted several actions whereby *SAA1* may affect tumour progression, including promoting secretion of various interleukins and TNFα, acting as an ECM (extracellular matrix) adhesion protein, and mediating production of reactive oxygen species in neutrophils.

DMBT1 (deleted in malignant brain tumours 1) is a secreted glycoprotein that contains many scavenger receptor domains. It plays a role in intestinal mucosal defence against bacteria and preventing inflammation by binding to mucosal defence proteins and surfactant proteins that bind specific pathogen markers[200]. It may have role in inflammatory bowel disease[201,202].

5.4.2.1 Two-gene model for recurrence after adjuvant radiotherapy

The two-gene model for disease-free survival in patients that had adjuvant radiotherapy after TEM included increased *NUPR1* and *COL8A2*. The model classified 40% of patients as high risk, with 3-year disease-free survival of 68% compared to 100% for the low-risk majority. The small numbers available limit confidence in this model.

NUPR1 (nuclear protein 1, aka *COM-1*) is a small protein, expressed in response to stresses and has been found overexpressed in several cancers, particularly pancreatic. Acting in combination with various molecules it regulates the cell cycle, programmed cell death and transcription, and is required for the regulation of TGF-β activity[203]. With its multiple functions, it can act as both tumour promotor and suppressor. In colorectal cancer it shows greater expression in early stage tumours[204].

COL8A2 is a component of collagen, important in the extracellular matrix.

While this model is not to be relied on, it does hold promise that the expression levels of certain, not yet completely elucidated, genes could predict whether a rectal cancer that has been locally excised has a significant risk of recurring after adjuvant radiotherapy. If this information were available following local excision, the patient and clinician could come to a more informed decision about the most appropriate subsequent pathway, whether this be adjuvant radiotherapy, radical surgery or close surveillance.

5.4.3 Future directions

This chapter has found some promising prospects for better stratification of locally excised early rectal cancers. It confirms the distinction from colon cancers, as factors found prognostic in that setting such as ploidy and stromal and immune scores are not of value in this population of early rectal cancers. A simple measure of tumour stromal fraction has some value, more so when combined with ploidy status, but more sophisticated AI-based analysis of the same H&E slide can yield a superior prognostic marker. Absence of the three common mutations, *APC*, *TP53* and *KRAS*, is also information that is readily available where a mutation panel is routinely used and provides a good indication that an early rectal cancer may be more likely to recur and so be worthy of closer surveillance. Gene expression data takes more time and expense to obtain, but certain combinations of genes show a strong association with recurrence and should be explored further. This will be taken forward in the first instance by seeking to validate the gene expression findings in a larger tumour set from the S:CORT project[95]. These markers will also be incorporated into the proposed Vorates trial, discussed at the end of the previous chapter. Having the ability to inform patients after local excision that their individual tumour is more likely than not to recur, or to be sensitive to radiation, rather than just relying on historic predictions based on a few histopathology features, would enable better decisions to be made about subsequent management.

Chapter 6: Adenoma-carcinoma progression in early rectal cancer

6.1 Introduction

This chapter continues the investigation of tissue samples of locally excised early rectal cancer from patients in the TEM database described in Chapter 4. In a quarter of these samples, the section showed both the early cancer and the precursor advanced polyp from which the cancer was arising. This chapter looks in detail at differential characteristics of the adenoma and carcinoma regions to assess markers of the transformation and progression which may provide insights into mechanisms and potential therapeutic targets.

The differentiating feature of cancer compared to polyp is invasion through the muscularis mucosae into the submucosa. Here, there is a network of blood vessels and lymphatic channels which can allow the tumour not only to invade locally but also to metastasise to distant sites. By comparing features of the same tumour before and after this invasive transformation has taken place it should be possible to identify features that are important in this process.

Background for this chapter is contained in Section 1.3.

6.1.1 Aim

1) To identify significant pathways involved in the progression from adenoma to carcinoma in rectal cancer

6.2 Methods

All methodology is described in Chapter 2: Materials and methods.

6.3 Results

There were 44 samples (26% of total TEM samples) where both adenoma and carcinoma were present in the section. These are very early cancers with the precursor polyp still present and identifiable. The polyp and cancer regions of these tumours were examined separately to investigate any differential properties that may give an insight into early invasiveness, the differentiating feature of cancer compared to polyp. The polyp and cancer regions were marked on the H&E slide and the RNA was extracted separately from each part of the tumour (Figure 6.1).

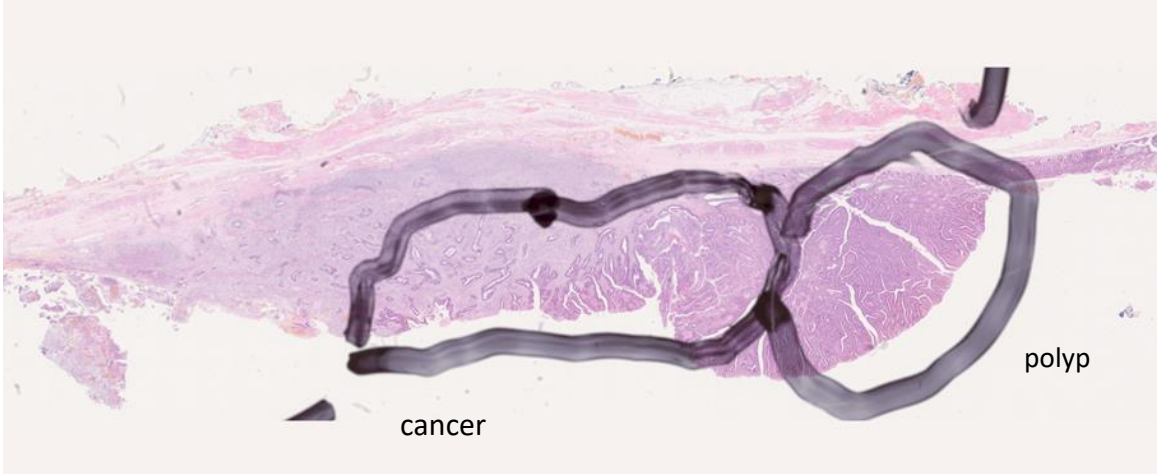


FIGURE 6.1: EXAMPLE OF A TISSUE SECTION ANNOTATED TO INDICATE CANCER AND POLYP REGIONS, WITH POLYP REGION ON THE RIGHT AND CARCINOMA REGION ON THE LEFT. THESE REGIONS WERE DISSECTED SEPARATELY FOR RNA AND DNA EXTRACTION**.**

6.3.1 Molecular scoring for compartments

DNN digital analysis was not available for the polyp component, but it was possible to use the ESTIMATE method[101] to infer the fraction of stromal and immune cells in the compartments using gene expression data for 40 paired samples. The stroma score was higher in the carcinoma part than the adenoma in almost all tumours (37/40, 93%, $p<0.0001$) whereas the immune score was mixed, increasing in the carcinoma part in 55% (22/40) and decreasing in the rest; paired t test showed no significant difference between regions, $p=0.41$ (Figure 6.2). This suggests an early

feature of the malignant transformation is expansion of the stromal compartment of the tumour whereas immune changes are inconsistent. The previous chapter demonstrated the importance of the immune infiltrate as a prognostic marker, but this finding suggests it is not so important in the malignant transformation.

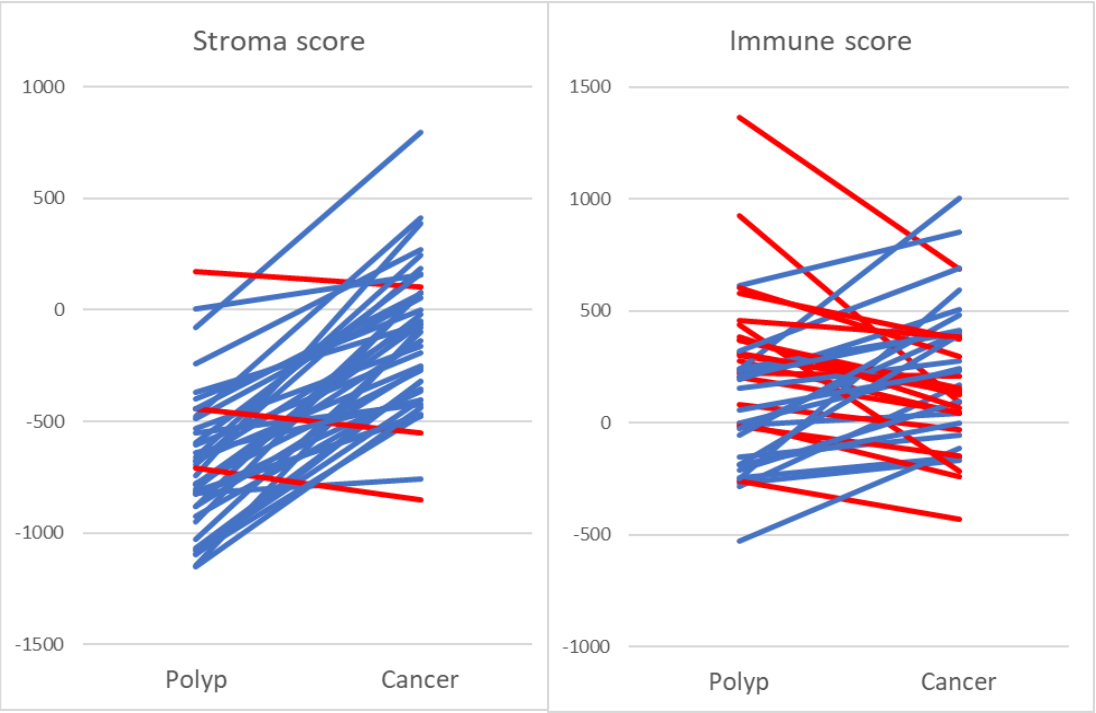


FIGURE 6.2: ESTIMATE[101] STROMA AND IMMUNE SCORES IN 40 PAIRED ADENOMA AND CARCINOMA REGIONS OF THE TUMOUR. A. ESTIMATE STROMA SCORE SHOWS INCREASE IN ALMOST ALL TUMOURS; BLUE INDICATES INCREASE IN SCORE, RED INDICATES DECREASE. PAIRED T TEST SHOWED A SIGNIFICANT DIFFERENCE BETWEEN POLYP AND CANCER PARTS, $P=2x10^{-13}$ **B.** ESTIMATE IMMUNE SCORE SHOWS NO CONSISTENT TREND BETWEEN POLYP AND CARCINOMA PARTS. PAIRED T TEST SHOWED NO DIFFERENCE BETWEEN THE PARTS, $P=0.41$.

6.3.2 Differentially expressed genes

Quality control assessment of the RNA sequencing data for the 44 pairs of samples suggested four paired samples may be outliers based on hierarchical clustering. The data and H&E slides for these were reviewed. In each case the H&E slide showed a good specimen and there were no exceptional clinical features. There was no indication of batch effect in the data. So, after consideration, these samples were retained in the cohort.

Preliminary analysis of the RNA sequencing data for the 44 pairs of samples yielded 9,804 genes, of which 2,147 were significant at the 5% FDR level. These included 1,097 genes that were more highly expressed in the carcinoma than the adenoma, and 1,050 genes with lower expression in the carcinoma region. Several methods were used to analyse the differentially expressed genes to discover the pathways and functional groupings involved. Genes more highly expressed in the carcinoma region are considered first, then those more highly expressed in the adenoma region.

6.3.3 Genes enriched in the carcinoma region

6.3.3.1 Gene ontology

This analysis considers the genes that were differentially expressed between the adenoma and carcinoma regions. Overall, the top ten enriched gene ontologies (GO) in the carcinoma regions included five cellular components (CC), four biological processes (BP) and one molecular function (MF). These are shown in Table 6.1; most ontologies deal with the extracellular matrix (ECM) or extracellular region.

TABLE 6.1: TOP 10 ENRICHED GENE ONTOLOGIES IN CARCINOMA REGION COMPARED WITH ADENOMA

Term	Type[1]	Total genes in term	Upwardly expressed genes in dataset		Downwardly expressed genes in dataset	
			No. genes	p value	No. genes	p value
ECM organization	BP	334	96	3.3E-45	14	0.68
Extracellular structure organization	BP	387	100	1.6E-42	17	0.61
Biological adhesion	BP	1,345	162	1.1E-24	84	0.0026
Cell adhesion	BP	1,338	160	4.8E-24	84	0.0023
Extracellular region	CC	4,295	399	2.2E-37	238	0.00067
Extracellular space	CC	3,051	303	7.1E-32	199	9.6E-08
ECM	CC	468	122	2.4E-52	21	0.58
Extracellular structure	CC	3,312	347	1.3E-42	208	7.7E-07
Collagen-containing ECM	CC	399	114	2.2E-53	20	0.38
ECM structural constituent	MF	158	62	2.0E-38	7	0.60

[1]BP=biological process, CC= cellular component, MF=molecular function

Using the DAVID online tool, the enriched ontologies were grouped into functional clusters (Table 6.2). Again, the ECM and its components features most strongly.

TABLE 6.2: FUNCTIONAL CLUSTERS OF ENRICHED ONTOLOGIES IN THE CARCINOMA REGION DETERMINED BY DAVID TOOL

DAVID: functional clusters of enriched ontologies	Enrichment score
ECM organisation – including ECM-receptor interaction, focal adhesion and PI3K-Akt signalling pathway*	10.82
Extracellular region and secreted products	9.35
Collagen, especially collagen catabolic process	9.24
Cell cycle	6.26
Growth factor terms, especially insulin-like GF and epidermal GF	5.32

*The PI3K-Akt signalling pathway promotes metabolism, proliferation, cell survival, growth, and angiogenesis

6.3.3.2 Gene set enrichment analysis

This analysis considered all genes in the set rather than just the differentially expressed ones, with genes ranked in order of log fold change. As only genes with Entrez identifiers could be used, this analysis covered 9,567 (97.6%) of the available genes.

Both Hallmark and Reactome pathways were assessed. Using the Molecular Signatures Database Hallmark gene sets, there were 17 significantly enriched gene sets. Using the Reactome gene sets, there were 25 pathways enriched in the carcinoma region with the lowest adjusted p value (0.00064). Many of the pathways showed overlap, so the Collapse pathways function was used to obtain a list of independent pathways. The pathways from both gene sets with a normalised enrichment score over 2 are shown in Table 6.3; again the ECM features prominently.

TABLE 6.3: HALLMARK AND REACTOME PATHWAYS MOST ENRICHED IN THE CARCINOMA REGION

	Adjusted p value[1]	Normalised enrichment score[2]
Hallmark pathway		
Epithelial mesenchymal transition	8.7E-05	3.15
Coagulation	8.7E-05	2.29
E2F targets (cell cycle)	8.7E-05	2.26
Angiogenesis	8.7E-05	2.26
Interferon gamma response	8.7E-05	2.02
Apical junction (cytoskeleton)	8.7E-05	2.01
Reactome collapsed pathways		
ECM organization	0.00061	2.96
Collagen degradation	0.00061	2.73
Integrin cell surface interactions	0.00061	2.67
Collagen biosynthesis & modifying enzymes	0.00061	2.64
ECM proteoglycans	0.00061	2.55
Collagen chain trimerization	0.00061	2.52
Insulin-like growth factor transport & uptake by IGF binding proteins	0.00061	2.34
Signalling by PDGF	0.00061	2.32
MET activates PTK2[3] signalling	0.00061	2.22
Chondroitin sulphate/ dermatan sulphate metabolism	0.00096	2.11

[1]p value adjusted to correct for false discovery rate, [2]Normalised enrichment score = ES normalised to mean enrichment of random samples of same size, [3] PTK2= focal adhesion kinase.

6.3.3.3 KEGG pathway analysis

KEGG pathway analysis also uses the differentially expressed genes and analysed the 2,112 with Entrez identifiers. Of the 333 pathways returned, 28 had p value <0.01 for differential expression. The 10 most significant pathways are shown in Table 6.4; these include differential expression in both directions. Figure 6.3 shows a graphical arrangement of the most significant pathways and associations, with expression of the relevant genes annotated by Entrez ID.

TABLE 6.4: TOP 10 MOST SIGNIFICANT KEGG PATHWAYS

Pathway	No. genes in pathway	No. genes in dataset	p value for enrichment
ECM-receptor interaction	86	30	1.5E-08
Proteasome	45	18	1.2E-06
Focal adhesion	199	44	2.0E-05
Protein digestion and absorption	90	25	2.5E-05
TGF-β signalling pathway	94	25	5.6E-05
AGE-RAGE signalling pathway in diabetic complications	100	26	6.0E-05
Amoebiasis	95	24	0.00019
DNA replication	36	12	0.00054
Fatty acid degradation	44	16	2.0E-05
Mineral absorption	53	15	0.00083

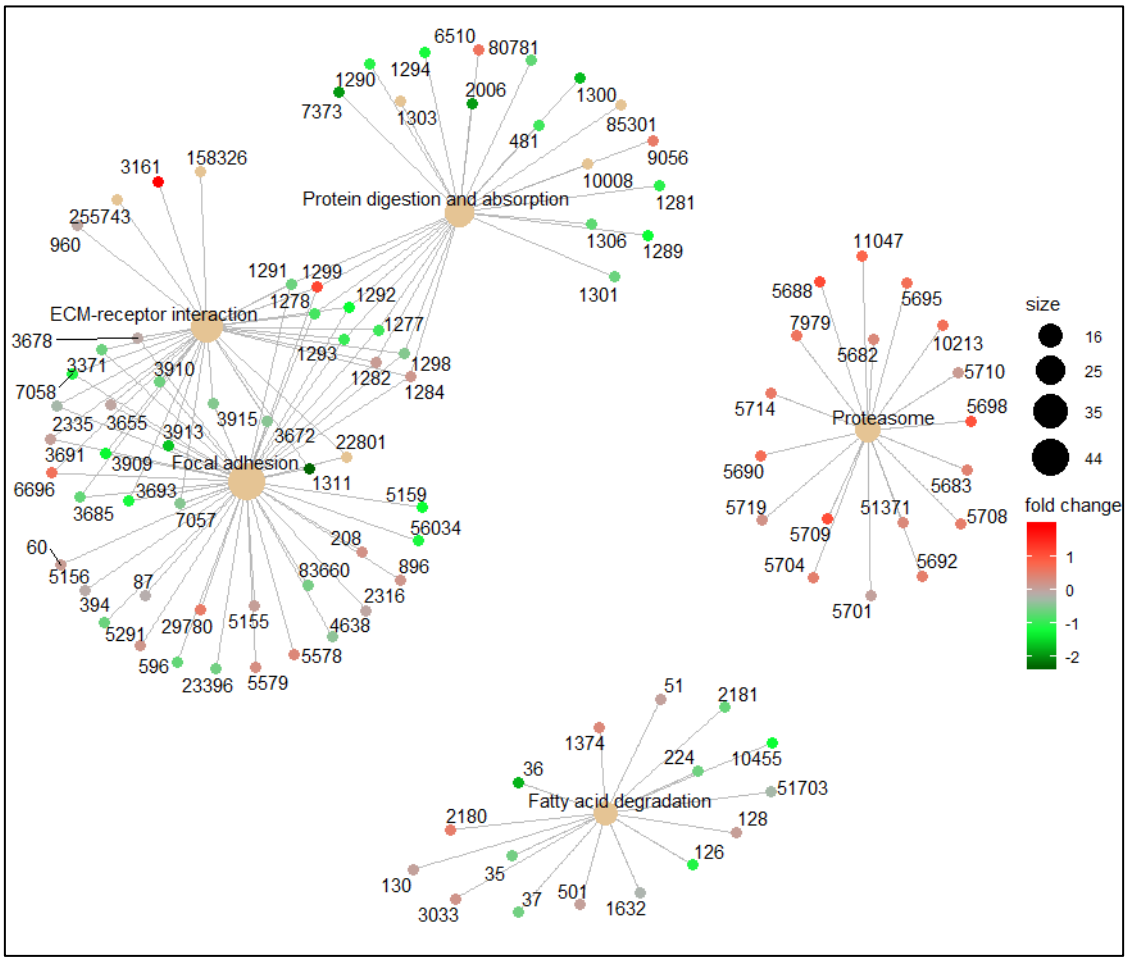


FIGURE 6.3: KEGG PATHWAYS, SHOWING THE MOST SIGNIFICANT DIFFERENTIALLY EXPRESSED PATHWAYS. NUMERICAL ANNOTATIONS ARE GENE ENTREZ IDENTIFIERS, RED COLOUR INDICATES ENRICHMENT IN THE CARCINOMA REGIONS AND GREEN INDICATES ENRICHMENT IN ADENOMA REGIONS.

6.3.3.4 Protein-protein interaction network analysis

Protein-protein interaction (PPI) analysis was also undertaken. This uses only the differentially expressed genes and can use the Ensembl identifiers, so all 2,147 DE genes were used. The top 400 were used to create a STRING network. The resultant network had 400 nodes, with 4,761 edges compared with an expected 1,992, giving a highly significant p value for PPI enrichment, <0.0001. The average node degree was 23.8 and the average local clustering coefficient was 0.335. The diagram for this can be accessed at http://version10.string−db.org/10/p/8235458323.

For these top 400, the gene ontologies and KEGG pathways were the same as previously identified. Using the PFAM protein domains, two significant pathways were detected, and eight pathways from the INTERPRO protein domains were detected. These are shown in Table 6.5; most relate to ECM components.

TABLE 6.5: PFAM AND INTERPRO PROTEIN DOMAINS SIGNIFICANTLY DIFFERENTIALLY EXPRESSED BETWEEN ADENOMA AND CARCINOMA REGIONS

Pathway description	count in gene set	FDR*
Tissue inhibitor of metalloproteinase	3	0.02
Insulin-like growth factor binding protein	4	0.04
Netrin domain[1]	5	0.00095
Tissue inhibitor of metalloproteinases-like	5	0.0011
Protease inhibitor I35 (TIMP)	3	0.0095
Proteinase inhibitor I35b (TIMP), C-terminal	3	0.0095
Tissue inhibitor of metalloproteinase, conserved site	3	0.0095
Insulin-like growth factor-binding protein	4	0.025
C-type lectin fold	6	0.025
Insulin-like growth factor binding protein, N-terminal	5	0.045

*FDR = false discovery rate, [1]This domain is found in compounds with roles in axon guidance, Wnt signalling regulation and metalloproteinase activity control.

The common features here are IGF (Insulin-like growth factor) binding proteins and TIMPs (tissue inhibitors of metalloproteinases) that inhibit the MMPs (matrix metallopeptidases).

Using the top 800 differentially expressed genes that could be mapped to genes in the String database, three main clusters of protein interactions were obtained (Table 6.6). These include groupings differentially expressed in both directions.

TABLE 6.6 THREE MAIN CLUSTERS OF DIFFERENTIALLY ENRICHED PROTEINS DETERMINED BY PPI ANALYSIS

PPI: clusters of enriched protein interactions	No. proteins	Interactions (expected)	p value
ECM matrix proteins	221	4,606 (563)	0
Metabolic processes and binding, specifically: **- fatty acid metabolism and degradation** **- aldosterone-regulated sodium reabsorption** **- fructose and mannose metabolism**	368	4,448 (3,486)	0
Cellular Component gene ontologies, specifically: **- membrane components, extracellular parts, vesicles and cytoplasm**	153	587 (116)	0

Diagrams for these clusters can be seen at http://version10.string−db.org/10/p/5035458319, version10.string−db.org/10/p/1078459738, version10.string−db.org/10/p/1100459739

Figure 6.4 summarises the processes most enriched in each region. All those most enriched in the carcinoma region concern the ECM, while the adenoma region features many normal metabolic processes including fatty acid oxidation, cellular response to metal ions and negative growth regulation.

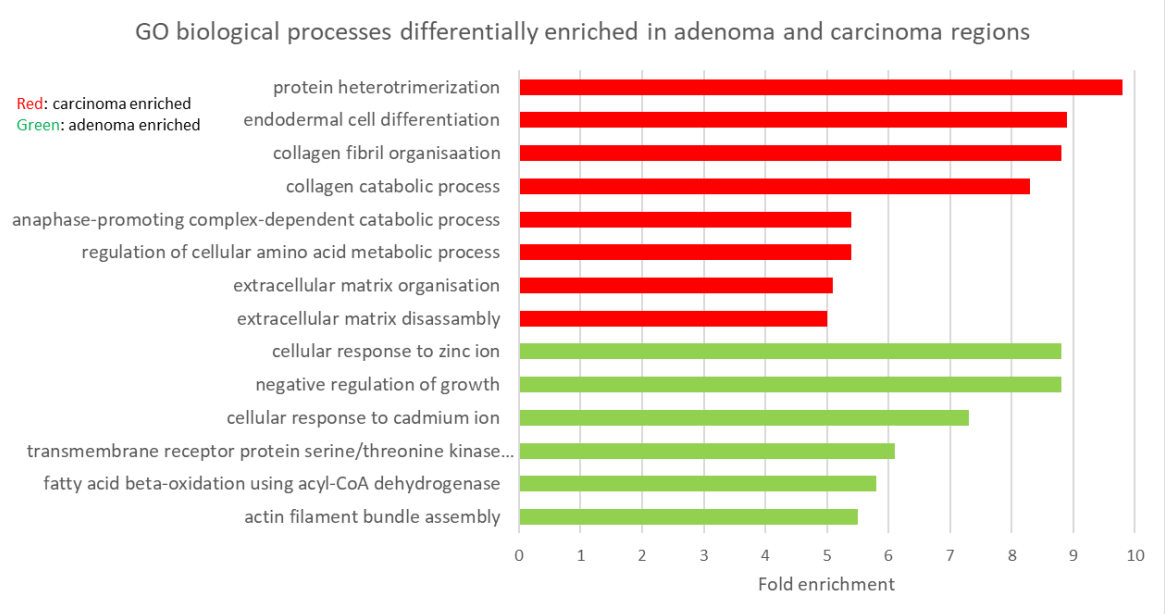


FIGURE 6.4: BIOLOGICAL PROCESSES MOST DIFFERENTIALLY EXPRESSED IN EACH REGION, SHOWING PROCESSES WITH A FOLD CHANGE >5. RED INDICATES MOST ENRICHED IN THE CARCINOMA REGION, GREEN MOST ENRICHED IN THE ADENOMA REGION.

6.3.3.5 Summary of genes most enriched in the carcinoma region

The common features that show up across the different techniques used to explore the genes with increased expression in the early carcinoma region compared to the advanced adenoma are:

- Extracellular matrix, including secreted products, interactions with receptors, collagen synthesis and most prominently, collagen degradation
- Focal adhesion, including PTK2 (= focal adhesion kinase) signalling
- Angiogenesis
- Growth factors, especially IGF, Epidermal Growth Factor (EGF) and platelet derived growth factor (PDGF).
- Signalling pathways
- Cell cycle

The most prominent are explored in more detail below.

EXTRACELLULAR MATRIX

The ECM provides structure and forms a major component of the microenvironment so can affect cellular activities such as proliferation, adhesion, migration, differentiation and cell death[205].

COLLAGEN FORMATION AND DEGRADATION: Collagen formation involves biosynthesis of chains, trimerisation of chains and then assembly of the fibrils into fibres and networks. These pathways are significantly over-expressed in the carcinoma regions. There are 22 significantly differentially expressed collagen genes, of which 21 are more highly expressed in the carcinoma. Figure 6.5A shows the differentially expressed collagen genes and other genes involved in its formation, including PLODs, encoding lysyl hydroxylase, *LEPREL2*, a hydroxylase, *SERPINH1* for folding of procollagen, *ADAMTS 2* and *14*, *BMP1* and *PCOLCE*, proteinases that activate procollagen, and several cathepsins (CTS) and matrix metallopeptidases (MMP) which aid fibril assembly into networks. The laminin that anchors collagen fibrils to the basement membrane (*LAMA3*) is under-expressed.

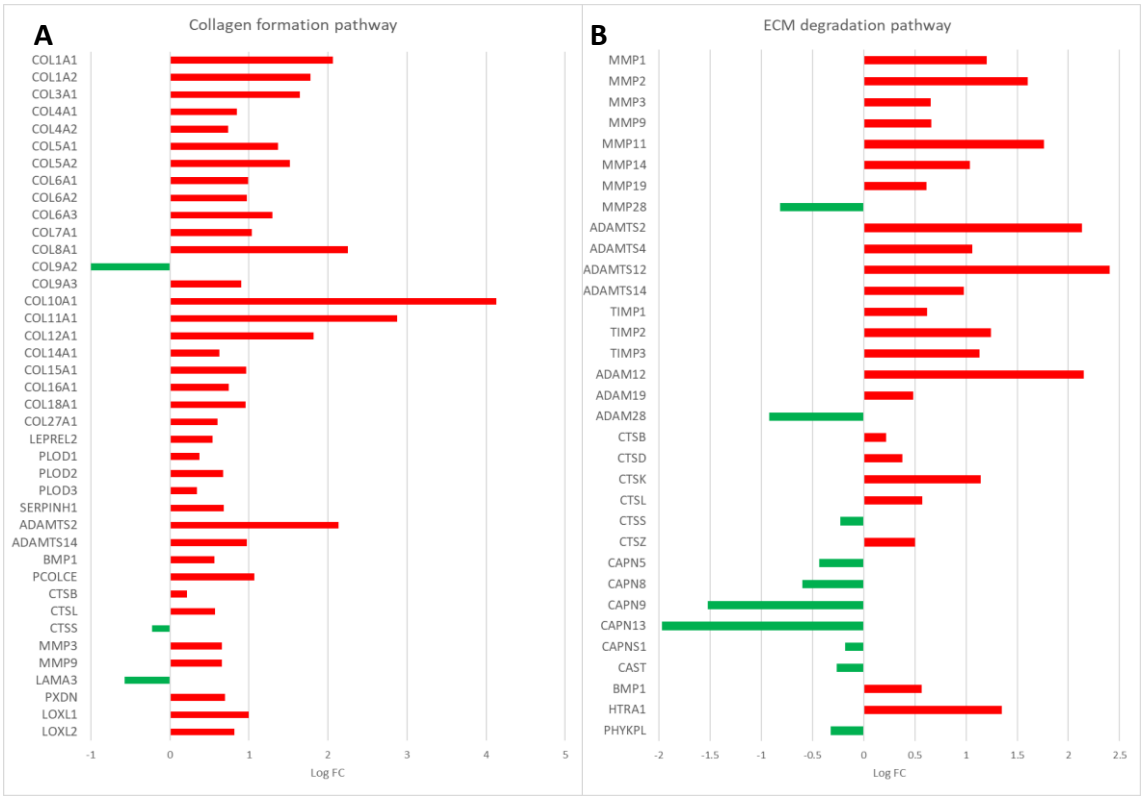


FIGURE 6.5: DIFFERENTIALLY EXPRESSED GENES IN THE ECM INVOLVED IN COLLAGEN FORMATION AND DEGRADATION. A. GENES INVOLVED IN COLLAGEN FORMATION PATHWAYS. FOLD CHANGE IS SHOWN ON THE AXIS. RED INDICATES OVER-EXPRESSION IN THE CARCINOMA REGION, GREEN INDICATES OVER-EXPRESSION IN THE ADENOMA REGION. **B.** GENES INVOLVED IN ECM AND COLLAGEN DEGRADATION PATHWAYS.

There are two main families of ECM remodelling enzymes which breakdown collagen and other ECM components. Eight members of the MMP and four members of the ADAMTS family are significantly over-expressed. Figure 6.5B shows the expression of these and other proteases significantly differentially expressed, including the tissue inhibitors of metalloproteinases (TIMPs) and CTSs. Interestingly the calpain (CAPN) family of proteases, and their regulator, *CAST*, are significantly more expressed in the adenoma regions. At least two of these, *CAPN8* and *CAPN9* are thought to be associated with colonic polyp development[206]. It seems clear that breakdown and reforming of the ECM is a critical factor in the transition from benign adenoma to invasive carcinoma with expansion of the desmoplastic component. Skhinas and Cox[207] note that the remodelling involves post-translational collagen crosslinking which makes the tumour microenvironment stiffer and more fibrotic, and this remodelling appears to not merely accompany

tumour growth but to drive cancer progression by activating intracellular kinase signalling pathways.

OTHER ECM COMPONENTS: Figure 6.6 shows gene expression plots for other ECM pathways. Several proteoglycans are over-expressed; these have roles in collagen formation and stabilisation and also stimulate angiogenesis and neuron development.

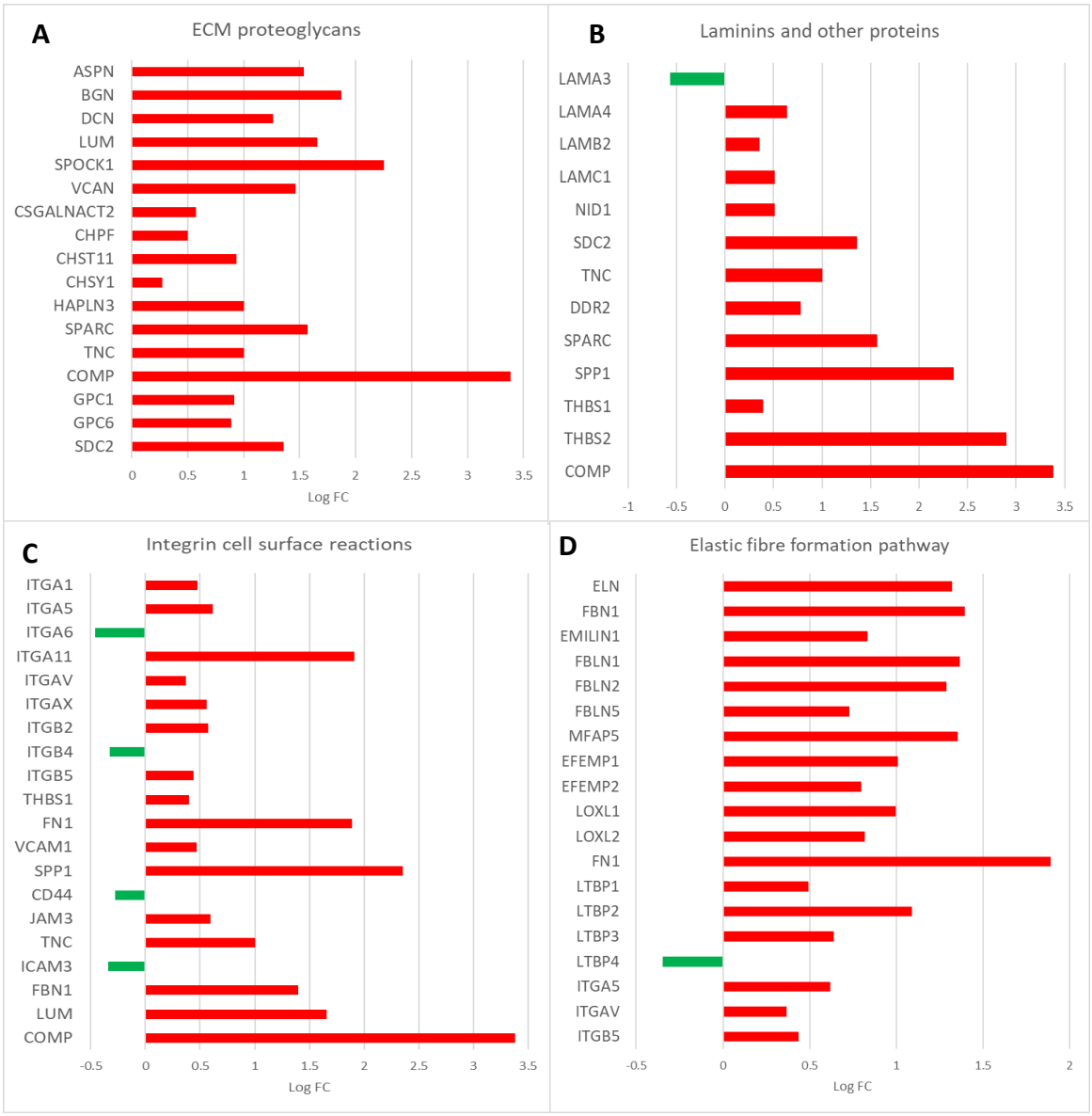


FIGURE 6.6: DIFFERENTIALLY EXPRESSED GENES INVOLVED IN VARIOUS ECM COMPONENTS. A. PROTEOGLYCANS. FOLD CHANGE IS SHOWN ON THE AXIS. RED INDICATES OVER-EXPRESSION IN THE CARCINOMA REGION, GREEN INDICATES OVER-EXPRESSION IN THE ADENOMA REGION. **B.** LAMININS AND RELATED PROTEINS. **C.** INTEGRIN CELL SURFACE REACTIONS. **D.** ELASTIC FIBRE FORMATION PATHWAY.

The lamimins and related proteins form part of the basement membrane and bind to a wide variety of ECM components, growth factors and cytokines, and so affect cell differentiation, migration, and adhesion. Integrins are cell surface receptors that allow cell adhesion to the ECM; many of these are also over-expressed. Some of these are important in leucocyte function and migration and so have a role in the inflammatory response. Genes involved in the formation and degradation of elastic fibres are also over-expressed; these have a role in binding and sequestering growth factors such as TGF-β into the ECM. Three of the latent TGF-β binding proteins (LTBPs) are significantly over-expressed in the carcinoma regions.

CANCER-ASSOCIATED FIBROBLASTS (CAF): CAFs are a major cellular component of tumour stroma and are responsible for the epithelial-mesenchymal transition signal in the carcinoma region[208] noted in Table 6.3. Li *et al.*[209] identified two distinct subpopulations of CAF in colorectal cancer, each with specific marker genes: CAF-A express genes related to ECM remodelling, including *MMP2*, the activator of TGF-β, while CAF-B express cytoskeletal genes and other markers of activated myofibroblasts. The differentially expressed genes indicate that the CAF-A subtype predominates in the carcinoma region in these early cancers (Figure 6.7).

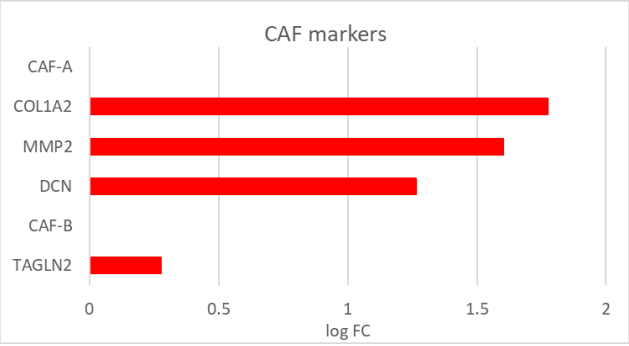


FIGURE 6.7: DIFFERENTIALLY EXPRESSED MARKER GENES FOR THE TWO TYPES OF COLORECTAL CANCER CAFS (CANCER-ASSOCIATED FIBROBLASTS).

FOCAL ADHESIONS

This is another pathway that consistently shows up as significantly enriched in the carcinoma region. These are assemblies of molecules that transmit signals between cells and the ECM,

containing integrins and other proteins. They also serve as anchors for cells, so when cells are migrating, they constantly form on one side and disassemble on the other. The over-expression in the carcinoma region may indicate increased activity because cells are migrating and invading as well as increased signalling. The increased expression of the Reactome pathway MET activates PTK2 (also known as Focal adhesion kinase) signalling is part of this function.

ANGIOGENESIS

Only in the analysis of Hallmark pathways does angiogenesis feature in the most differentially expressed pathways. However, many of the ECM components have a role in angiogenesis. Of the known matrix-derived endogenous inhibitors of angiogenesis the majority are significantly enriched in the carcinoma region, along with their integrin receptors. These include fragments of certain collagens, such as endostatin (from collagen XVIII), fragments of fibronectin and various fibulins and thrombospondins. On the other hand, most of the main stimulators of angiogenesis are not significantly enriched in the carcinoma region compared to the adenoma. For example, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are not differentially expressed. This suggests that while angiogenesis is important in the development of the adenoma, in the transition between adenoma and carcinoma, new blood vessel development is not such an important factor as this has already been initiated.

GROWTH FACTORS

Several growth factor pathways are overexpressed. A number of components of the IGF pathway are over-expressed in the carcinoma region including IGF2 and several binding proteins, while some proteases that specifically degrade the binding proteins are under-expressed (Figure 6.8A). The IGFBPs may concentrate IGF at its receptors and they also have IGF-independent actions on the cell cycle and apoptosis. IGFBP3 interacts with TGF-β signalling[210]. The IGF system has a critical role in colorectal cancer development[211] and *IGF2* expression correlates with tumour progression and poor prognosis[212]. IGF2 promotes cell proliferation and ECM adhesions[213]. Belharazem *et al.*[214]

noted that IGF2 is a powerful driver of cell proliferation through the action of AKT1, and contributes to Wnt signalling through activation of WNT5a, which is significantly over-expressed in the carcinoma region. IGF2 and AKT1 have thus been proposed as potential therapeutic targets; antibodies[215], scavenger receptors[216] and IGF receptor inhibitors[217] have been investigated with some early success.

Several components of the PDFG pathway are also over-expressed in the carcinoma region (Figure 6.8B). These include two of the four PDGF chains, their receptors, ECM components they interact with, and downstream molecules activated by PDGF. Together these stimulate growth and motility in cells in the connective tissue including angio- and neuro-genesis and can promote immunosuppression and tumour survival.

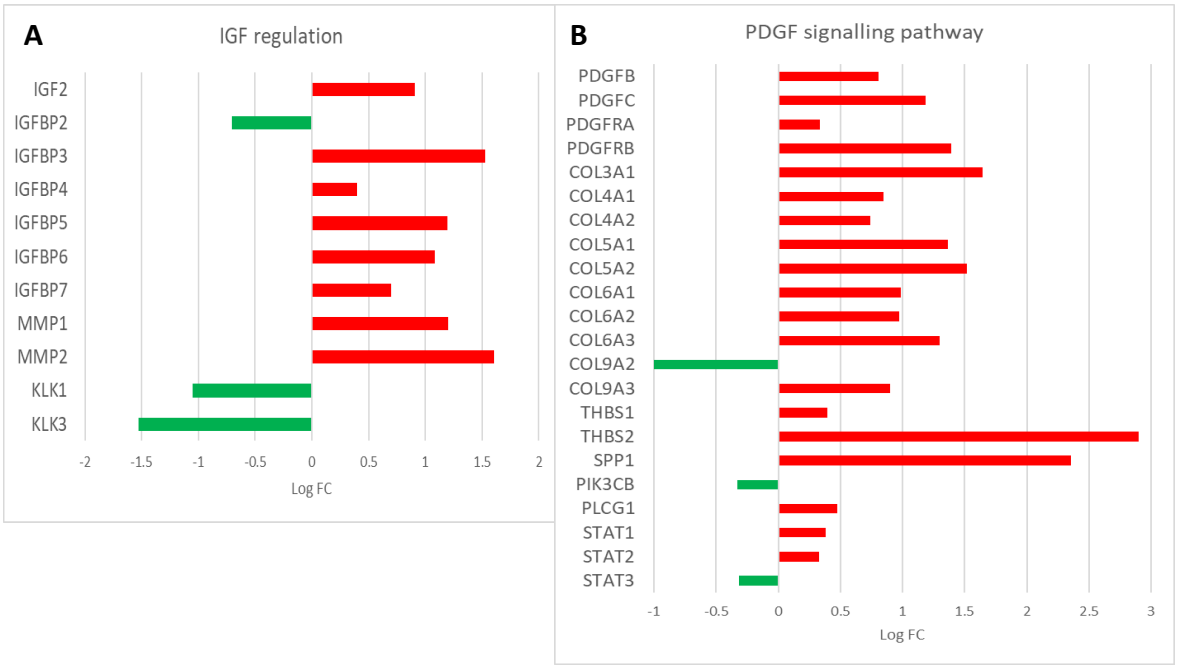


FIGURE 6.8: DIFFERENTIALLY EXPRESSED GENES INVOLVED IN IGF REGULATION AND PDGF SIGNALLING. A. INSULIN-LIKE GROWTH FACTOR(IGF) AND ITS REGULATORS THAT ARE DIFFERENTIALLY EXPRESSED. FOLD CHANGE IS SHOWN ON THE AXIS. RED INDICATES OVER-EXPRESSION IN THE CARCINOMA REGION, GREEN INDICATES OVER-EXPRESSION IN THE ADENOMA REGION. **B.** GENES INVOLVED IN THE PLATELET-DERIVED GROWTH FACTOR (PDGF) PATHWAY.

SIGNALLING PATHWAYS

The over-expressed PI3K/AKT/mTOR is a component of ECM re-organisation and plays a role in regulating the cell cycle, reducing apoptosis, and promoting cell proliferation. Other over-expressed pathways include mTORC1, which promotes protein production, KRAS, which promotes cell proliferation and differentiation, and MET, which promotes cell motility.

Of the various TGF-β signalling sub-pathways, signalling by activin (*INHB-A*&*B*) is significantly over-expressed; these are cytokines with wide-ranging effects including immune responses and cell proliferation, differentiation, and death. Other cytokines that are increased are the interleukins IL-4 & 13; their expression leads to downregulation of genes that promote inflammation and upregulation of genes involved in resolving the inflammatory process. This indicates a role for a changing immune microenvironment in the progression from adenoma to carcinoma, although the ESTIMATE scores did not show any consistent increase in the fraction of immune cells in the carcinoma. Through mathematical modelling, Gatenbee *et al*.[218] showed that the transition from adenoma to carcinoma is associated with remodelling of the immune environment with a shift from high cytotoxic T cell and low macrophage abundance in the adenoma, to high macrophage and neutrophil with low cytotoxic T cells as the tumour becomes malignant, although the present results cannot confirm this.

One subpathway within the Wnt signalling pathway is significantly under-expressed in the carcinoma region: the degradation of β-catenin (CTNNB1), an important protein in cell adhesion and gene transcription. This effect may promote Wnt signalling.

CELL CYCLE

Several sub-pathways in the cell cycle involve genes that are differentially expressed. These include DNA replication (Figure 6.9A) where many components are over-expressed in the carcinoma, and control of transition between phases in the cell cycle, into the S (DNA synthesis) phase, and then into the G2 phase when mitosis occurs. There are checkpoints in the cycle where the cell checks

the DNA is undamaged. At the G1/S checkpoint, the check involving p53 is significantly less expressed in the carcinoma compared to the adenoma region, indicating impaired checkpoint function which can allow replication of damaged templates.

APOPTOSIS

The intrinsic pathway for apoptosis, involving the BCL2 family, is differentially expressed. Genes that act promote apoptosis are over-expressed while those that inhibit apoptosis are less expressed than in the adenoma regions (Figure 6.9B).

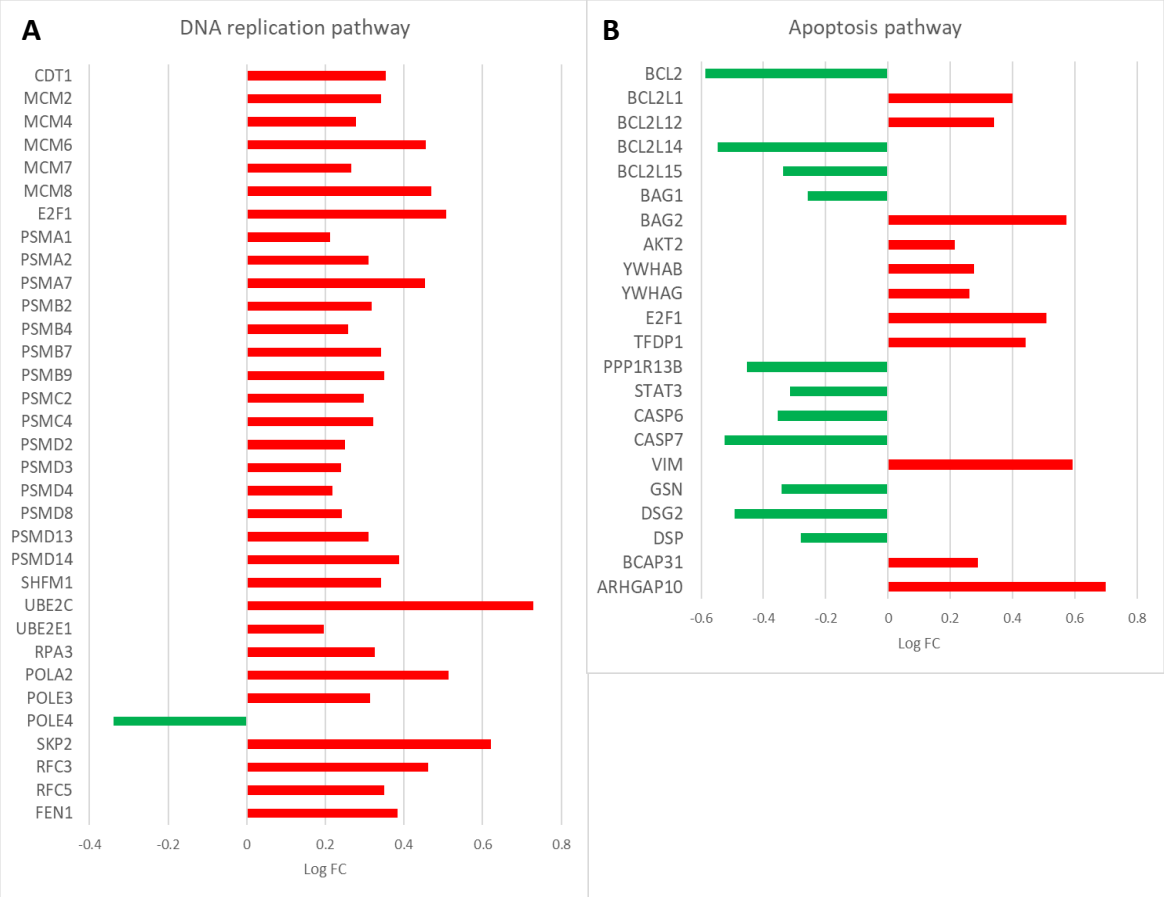


FIGURE 6.9: DIFFERENTIALLY EXPRESSED GENES INVOLVED IN DNA REPLICATION AND APOPTOSIS. A. GENES INVOLVED IN THE DNA REPLICATION PATHWAY. FOLD CHANGE IN SHOWN ON THE X-AXIS. RED INDICATES OVER-EXPRESSION IN THE CARCINOMA REGION, GREEN INDICATES OVER-EXPRESSION IN THE ADENOMA REGION. **B.** GENES INVOLVED IN THE APOPTOSIS PATHWAY

6.3.4 Genes enriched in the adenoma region

The gene ontologies that were most enriched in the adenoma region compared to the carcinoma were also identified and are shown in Table 6.7.

TABLE 6.7: TOP 10 ENRICHED GENE ONTOLOGIES IN ADENOMA REGION COMPARED WITH CARCINOMA

Term	Type[1]	Total no. genes in term	Upwardly expressed genes in dataset		Downwardly expressed genes in dataset	
			No. genes	p value	No. genes	p value
regulation of biological quality	BP	3,662	273	4.3E-11	231	1.0E-07
lipid catabolic process	BP	300	14	0.70	37	5.0E-08
drug metabolic process	BP	792	53	0.035	72	2.7E-08
oxoacid metabolic process	BP	1,091	100	1.8E-08	89	9.6E-08
carbohydrate derivative metabolic process	BP	1,169	94	1.5E-05	94	8.0E-08
extracellular space	CC	3,051	303	7.1E-32	199	9.6E-08
endomembrane system	CC	4,214	352	5.5E-23	262	3.4E-08
membrane	CC	9,049	503	0.019	497	3.61E-08
organelle membrane	CC	2,734	179	0.00049	183	6.06E-08
oxidoreductase activity	MF	662	39	0.23	65	7.04E-09

[1]BP=biological process, CC= cellular component, MF=molecular function

These cover five biological processes, four cellular components and one molecular function. In general, the biological processes are concerned with normal cellular metabolism.

Using DAVID, the enriched ontologies were grouped into functional clusters. The three most enriched clusters are shown in Table 6.8.

TABLE 6.8: FUNCTIONAL CLUSTERS OF ENRICHED ONTOLOGIES IN THE ADENOMA REGION DETERMINED BY DAVID TOOL

DAVID: functional clusters of enriched in adenoma ontologies	Enrichment score
oxidoreductase activity	5.56
metal binding and mineral absorption	4.75
fatty acid and lipid metabolism	3.78

For the Reactome pathways most enriched in the adenoma region, four had adjusted p values <0.01, see Table 6.9.

TABLE 6.9: REACTOME PATHWAYS MOST ENRICHED IN THE ADENOMA REGION

Reactome pathway	Adjusted p value[1]	Normalised enrichment score[2]
O-linked glycosylation of mucins	0.0010	2.10
Phase I - Functionalization of compounds	0.0079	1.85
Biological oxidations	0.0029	1.81
Transport of small molecules	0.0022	1.52

[1]p value adjusted to correct for false discovery rate, [2]Normalised enrichment score = ES normalised to mean enrichment of random samples of same size.

On PPI, the cluster involving metabolic processes and binding includes several proteins that were over-represented in the adenoma regions. Among the ten with the greatest fold change are myoglobin, two calpain proteases, REG4, involved in the inflammatory and metaplastic responses of the gastrointestinal epithelium, and DUOX2 which generates hydrogen peroxide and plays a role in antimicrobial defence at the mucosal surface. This suggests a loss of some of the normal protective mechanisms of the epithelial cells in the carcinoma region.

6.3.4.1 Summary of genes most enriched in the adenoma region

The common features that show up across the different techniques used to explore the genes with increased expression in the adenoma region compared to the carcinoma are:

- Fatty acid degradation
- Mineral absorption
- Mucin glycosylation
- Oxidation / oxidoreductase activity

Some of these are explored in more detail below.

MUCIN GLYCOSYLATION

One pathway which is significantly more expressed in the adenoma region is glycosylation of mucins. Mucins function in cell signalling as well as barriers and lubricants; glycosylation serves to

activate them. There are some 20 genes that encode mucins, eight of which are differentially expressed, all reduced in the carcinoma region. Several other enzymes involved in the glycosylation process are also reduced (Figure 6.10A). This indicates reduction in mucin production, with reduced mucosal defence and cell signalling as part of the transformation from adenoma to carcinoma. Krishn *et al.*[219] found a significant shift during the adenoma-carcinoma progression with reduced MUC 2 and 4, as found here, but increased MUC 1 and 5AC, in contrast to the present findings. However, their study was based on unmatched colon tissue arrays comparing 16 cancers and 30 assorted polyp types which may make their results less precise.

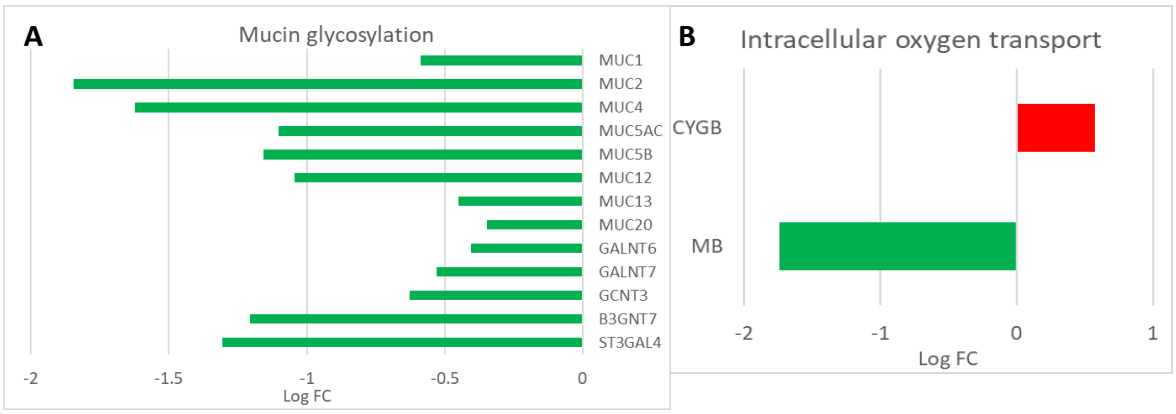


FIGURE 6.10: DIFFERENTIALLY EXPRESSED GENES INVOLVED IN MUCIN GLYCOSYLATION AND INTRACELLULAR OXYGEN TRANSPORT. A. GENES INVOLVED IN MUCIN GLYCOSYLATION. FOLD CHANGE IN SHOWN ON THE X-AXIS. RED INDICATES OVER-EXPRESSION IN THE CARCINOMA REGION, GREEN INDICATES OVER-EXPRESSION IN THE ADENOMA REGION. **B.** GENES INVOLVED IN INTRACELLULAR OXYGEN TRANSPORT.

INTRACELLULAR OXYGEN TRANSPORT

This is the principal component of the 'transport of small molecules' pathway that is significantly differentially expressed. There are three types of globin that transport oxygen in the cytoplasm: myoglobin, neuroglobin, and cytoglobin. In the carcinoma region the myoglobin gene, *MB*, is less expressed while the cytoglobin gene *CYGB* is more expressed (Figure 6.10B). So there appears to be a shift in the prevalent oxygen transport molecule from myoglobin in the adenoma to cytoglobin in the carcinoma. Both molecules are involved in oxygen transport and scavenging free radicals including NO[220]. Myoglobin is found primarily in muscle and also epithelial cancer cells[221]. Flonta *et*

al's finding of increased myoglobin concentration in colon and other epithelial cancers contrasts with the present finding. Cytoglobin in found mainly in fibroblasts and smooth muscle cells of blood vessels and is known to have a tumour suppressor role[222] but there is little specific information regarding a role in colorectal cancer. Oleksiewicz *et al.*[222] noted that cytoglobin is upregulated in fibrosis and downregulated in several cancers. In the current study the strong stromal activity in the carcinoma region, with a fibroblast infiltrate, is the most likely explanation for the prominence of cytoglobin. This prominence in this setting suggests it may be worth consideration as a potential therapeutic target.

METABOLISM

Many of the genes less expressed in the carcinoma region reflect normal cellular metabolism, in particular oxidation, transport of ions across cell membranes and fatty acid metabolism. Cancer cells in general are known to employ fatty acid metabolism to generate energy when there is insufficient glucose so the reduction in fatty acid metabolism noted here suggests that in these early cancers energy substrates are not yet in short supply, although the reduction in normal oxidation processes could indicate diversion of the available oxygen to increased energy metabolism. Carvalho *et al.*[223] also noted reduced fatty acid metabolism in colorectal cancer samples compared to adenomas. It is interesting to note that the carcinoma regions have a low expression of *BCL2*, noted above in the apoptosis pathway. Low *BCL2* inhibits the activity of CPT1 (carnitine palmitoyl transferase 1), which is also low in the carcinoma region, and so prevents fatty acids entering the mitochondria to be processed[224]. The altered metabolism in the carcinoma also presents a potential therapeutic target as drugs that selectively starve cancer cells of their required nutrients can restrict growth and eventually kill cancer cells. Such drugs are generally less toxic than chemotherapy or radiation[225]. However, the present findings do not suggest that the altered

metabolism is sufficiently differentially expressed in these early rectal cancers to present a substantial target.

6.4 Discussion

This study has found many differentially expressed genes between adenoma and carcinoma. One advantage of this study over others is that the adenoma and carcinoma samples were matched, coming from the same tumour, so allowing a clearer signal from the genes. In addition, all the cancers were at any early stage and conversely all the adenomas were at an advanced stage and had malignant potential. Carvalho *et al.*[223] only found 248 significantly differentially expressed genes when comparing unpaired samples, so while they found gene sets associated with ageing and chromosomal instability were upregulated in the cancers and fatty acid metabolism was downregulated, consistent with the current study, the heterogeneity of their samples probably masked other significant pathways.

6.4.1 Extracellular matrix remodelling

The principal finding in this chapter is the over-expression in the carcinoma region of genes concerned with biological processes involving the ECM, collagen, and cellular adhesion. Remodelling of the ECM changes the microenvironment of the tumour cells and its prominence in this analysis indicates this altered microenvironment is a major feature in the progression from adenoma to carcinoma. The fact that this remodelling is not happening in the advanced pre-cancerous adenomas indicates that it is a consequence rather than a cause of the malignant transformation that allows epithelial cells to invade through the basement membrane.

The effect of the increased collagen, with more folding and cross-linking, and increased fibronectin is to stiffen the ECM surrounding the epithelial cancer cells. This increased stiffness is known to

influence the cells through the process of mechanotransduction whereby mechanical forces around the cell are converted into electrochemical activity[226]. Cells respond by altering gene expression to increase cell cycle activity, promote epithelial-mesenchymal transition (EMT) and enhance cell motility[227,228]. As epithelial cells lose their intercellular adhesions, they are freer to migrate through the ECM. When the ECM is stiff this is likely to provide more traction for the cell to migrate and squeeze through spaces in the basement membrane or vascular endothelium and so be able to invade and spread. So, while the ECM remodelling is not the cause of the initial invasive step, it rapidly progresses once the malignant transformation has taken place to maintain and promotes the invasive potential. A major player in the ECM remodelling are CAFs, which act to disrupt networks, and promote angiogenesis and invasion[229].

6.4.2 Cancer-associated fibroblasts

The ESTIMATE scores show a consistent increase in the fraction of stromal cells in the tumour in the carcinoma compared with adenoma. CAFs make up the major portion of these stromal cells. Kobayashi *et al.*[230] note four ways in which CAFs orchestrate cancer development: direct interaction with tumour cells by secreting tumour-promoting factors and physically pulling cells to guide migration; secretion of immunomodulators including TGF-β leading to immunosuppression; secretion of angiogenic factors; and ECM remodelling by synthesis and degradation which also increases ECF stiffness and promotes proliferation and invasion. The last is the most significant action apparent in this study.

It is not yet clear where the CAFs arise from, and whether the distinct subtypes (Figure 6.7) arise differently. Options for their origin include EMT from tumour epithelial cells, activation of normal fibroblasts or differentiation from circulating or local perivascular stem cells. It would seem feasible that the CAF-A type which express ECM remodelling genes and predominate in these early carcinoma regions, may arise from transdifferentiation from the epithelial cancer cells via EMT. If

the earliest malignant cells initiate the ECM remodelling that then promotes EMT, these same cells which have acquired the phenotypic increased ECM remodelling gene expression would then continue this expression as they become CAFs. This then becomes a cycle with the CAFs stiffening the ECM and promoting EMT which creates more CAFs. The CAFs have also been shown to promote invasion by directly pulling and stretching the basement membrane, which opens gaps through which the epithelial cells can migrate[227].

Another major finding of this chapter is the significantly increased expression of *IGF2* and several of the IGF binding proteins in the carcinoma regions. Unger *et al.*[231] demonstrated that CAFs are the major source of the increased IGF2 levels in colorectal cancers and promote cancer cell survival via PI3K/AKT signalling. They also showed that ECM remodelling mediated by IGF2 promotes invasiveness. It is interesting that this apparently distinct finding of over-expressed IGF2 confirms the pivotal role of CAFs in the transition from adenoma to carcinoma.

CAFs have been investigated as a potential therapeutic target[230] and targeted in early clinical trials[232]. The present results indicate that specific targeting of the CAF-A subtype, or of its ECM actions, for example by blocking lysyl oxidase which crosslinks collagen or depleting hyaluronic acid, may be more effective than has so far been achieved. Cancer-associated fibroblasts are clearly a key element in the acquisition of invasive capability and the progression from adenoma to carcinoma. There is still a lot to be learnt about CAFs in rectal cancer and this is an area that would benefit from further investigation in the future.

6.4.3 Future directions

The importance of the stroma in the early malignant transformation is clear. The work described here will be followed by further investigation of the stroma using immunohistochemistry to multiplex stain the sections using a number of stromal, as well as immune markers, to assess differential expression between the adenoma and carcinoma regions.

The differences studied in this chapter are those that differentiate an advanced adenoma from the early cancer into which it is transforming. The instant of malignant transformation has already taken place and the differences seen here are those that allow the early cancer to continue its malignant trajectory. Another aspect to be investigated is whether these advanced adenomas, with clear malignant potential, can be differentiated from earlier polyps that have not acquired, and may never acquire, malignant potential. Such a study would not be able to use matched samples but would use samples from benign adenomas and seek features that are differentially expressed compared with the advanced adenomas. This could potentially identify features of polyps that are still benign, but are about to transform, and so the patient could receive closer surveillance to ensure complete removal of the polyp.

Chapter 7: Radiotherapy and quality of life

7.1 Introduction and background

This chapter looks at neoadjuvant radiotherapy as an organ-preserving treatment in rectal cancer. A study designed to look for potential markers of tumour responsiveness to radiotherapy required the preliminary establishment of a rectal cancer biobank. Several people contributed to this work; see the Acknowledgements for further details. The chapter also considers the important issue of patients' reported functional outcomes and quality of life after rectal-preserving treatments.

7.1.1 Neoadjuvant radiotherapy

Neoadjuvant radiotherapy has become common practice prior to standard radical surgery when MRI predicts a risk of involved margins. This may lead to down-staging so a clear margin can be obtained at surgery, and is associated with reduced local recurrence, although no improvement in overall survival for moderate-risk tumours, but is also associated with significantly worse bowel and sexual function after surgery[233]. The Dutch TME trial randomised 1,861 patients with resectable rectal cancer to surgery alone or short-course radiotherapy then surgery[234]. At 10 years the local recurrence rate was 11% in the surgery alone compared to 5% in the group with pre-operative radiotherapy. Braendengen *et al.*[235] compared longer-course radiotherapy alone with chemo-radiation (CRT) and found a higher rate of clear margins at surgery in the CRT group, and a lower local recurrence rate: 5% compared with 7%. The ACPGBI guidelines[17] recommend either short-course radiotherapy followed by immediate surgery or CRT followed by delayed surgery for high-risk resectable rectal cancer.

A metanalysis including 13 trials[236] showed that increasing the interval between CRT and surgery significantly increased the rate of pathological complete response. Al-Sukhni *et al.*[237] reviewed over 23,000 patients in the US National Cancer Database who had neoadjuvant CRT followed by radical surgery from 2006 to 2011. A pathological complete response was observed in 23%. Factors that

correlated with complete response included lower T and N stage, longer delay to surgery and higher radiation dose. The dose-response relationship[238] led to the concept of Total Neoadjuvant Treatment (TNT) using ever-higher doses of radiation to gain control of the primary tumour[239].

The occurrence of complete response raises the question as to whether the radical surgery is always required. Some patients may achieve organ preservation by CRT alone with a 'watch-and-wait' follow-up strategy or by local excision of the residual or recurrent tumour after a complete, or near-complete, clinical response. A review of 'watch-and-wait'[240] noted the difficulty in assessing response, as clinical and pathological findings do not always match. The Dutch Colorectal Audit recorded 82 patients who had local excision after neoadjuvant treatment with (near-)complete response[167]; (near-)complete pathological response was noted in only 47%. The International Watch & Wait Database reported a local regrowth rate of 25% among 880 patients with an initial clinical complete response; most regrowth occurred within the first two years[148].

While some patients achieve sustained complete response, and others have a partial or temporary response, around 20% have no response. This group may experience a worse outcome by having a delay of several months before excisional surgery[241]. A reliable predictor of response would enable radiation to be reserved for those who would benefit from it and spare patients who are not sensitive from the potential morbidity of the radiation and the delay to definitive treatment.

7.1.2 Molecular predictors of radiation response

There is not yet any robust predictor of which tumours are radiosensitive. A systematic review[242] found 85 papers addressing prediction of pathological complete response to CRT in rectal cancer. They noted limited predictive ability of clinical and radiological measures and concluded that molecular profiling was the most likely means by which predictions could be made. The included studies focussed on biomarkers associated with DNA repair, proliferation, angiogenesis, and apoptosis. They note that such studies have not yielded any reliable or reproducible predictor. The

complexity of tumour response makes a simple mechanism for radiosensitivity unlikely, implying rather that several genes and pathways are involved. They believe that only large, multi-centre studies using robust methodologies are likely to yield useful answers. Seeing the lack of success in discovering any repeatable marker, Perez *et al.*[243] suggested giving up the search.

A more recent systematic review[241] has taken a different approach, using network mapping to detect common gene ontologies among the potential markers analysed in 74 eligible studies. Considering all investigated biomarkers under five subheadings, they found that markers with a role in the cell cycle had little value, while those concerned with response to stimulus, cell death and cellular metabolism showed some promise. While they did not find any clear markers, they suggest that future studies direct attention to biomarkers involved in the stress response and cellular metabolism ontologies, as being the most likely to contain useful markers.

In yet another approach, Isella *et al.*[69] investigated three stromal gene signatures: from CAF, leucocyte and endothelial stromal cells, for any association with resistance to radiotherapy. They did not find a significant association between the three signatures in an individual cancer, but for each of the three signatures alone, and together in a compound score, they found a significant association with radioresistance.

Yuzhalin *et al*.[244] also focussed on the stromal component by looking at the matrisome; an inventory of all proteins that make up the ECM[245]. Through genome analysis Yuzhalin *et al.* identified a core set of almost 300 proteins that comprise this matrisome including collagens, proteoglycans, and glycoproteins. Using publicly available databases to analyse the expression of the matrisome genes in several cancer types, including colorectal, they found a set of nine genes that were significantly upregulated across the range of adenocarcinomas (*Col11A1, Col1A1, BGN, Col10A1, COMP, MFAP2, MXRA5, AGRN, SPP1*). Over-expression of this gene set was significantly associated with worse disease-free and overall survival. In colorectal tumours over-expressing the nine gene set, they noted there was also over-expression of the gene sets governing epithelial–mesenchymal

transition (EMT), hypoxia, angiogenesis. On the other hand, there was no over-expression of the gene sets governing apoptosis or genomic instability. Building on this, they identified a separate but related group of matrisome genes that had some predictive value in colorectal liver metastases (Gordon-Weeks, unpublished data). One aim of this chapter is to assess whether this also has any predictive value for radiosensitivity in primary rectal cancers where hypoxia and angiogenesis may play a role.

7.1.3 Establishing a rectal cancer biobank

To look for molecular markers that may have predictive value it is necessary to obtain tissue from newly diagnosed rectal cancer, prior to treatment. This can be logistically difficult to achieve, with only a short window of opportunity, and when the diagnosis, subsequent management pathway and prognosis are all still unclear.

This project began by establishing a biobank for rectal cancer tissue and blood samples. This involved working with several teams and some experimentation to determine the most appropriate processes for identifying patients, obtaining samples, collecting and storing samples, collating relevant follow up data, and identifying returning patients so further samples could be collected at each visit.

7.1.4 Quality of life and functional outcomes

Quality of life and functional outcomes are a vital consideration in managing rectal cancer. There are several treatment pathways available, some which aim to preserve rectal function. But all pathways carry their own risks. Deciding how to proceed requires balancing the risks and benefits, including the subsequent quality of life. This may be particularly important at the two ends of the spectrum: the elderly where treatment risks are higher; and those with early screen-detected cancers who have minimal symptoms.

Three organ-preserving strategies for rectal cancer were mentioned in Chapter 4: TEM alone and radiation before or after TEM. A fourth strategy, 'watch-and-wait' after (near-)complete response to neoadjuvant radiation, is addressed in this Chapter. These are not alternate options that may be selected at the time of diagnosis, but they are options that may become suitable for a particular patient over the course of their treatment. Hence, they are not presented as equivalent, but are compared to learn about the functional effects of the different treatments.

Functional outcomes after radical surgery have been studied[246] but there is not much information regarding organ-preserving treatments. In a direct comparison of 62 age- and sex-matched patients after TEM and TME at a median of 28 months after surgery, overall scores of quality of life were similar, but the TEM group showed significantly fewer problems with bowel function and a trend towards better sexual function[247]. In an even smaller study of 35 patients[248] impaired function and quality of life were observed in all patients at one month after surgery. The impairment persisted at six months in the TME group only, and at 12 months there was no significant difference compared with baseline in either group. A systematic review[249] found little evidence regarding comparative functional outcomes after TEM or TME.

Several studies have assessed function before and after local excision as there was initially concern that the large diameter of the TEM scope may cause sphincter dysfunction. Looking specifically at TEM surgery, several studies have shown that ano-rectal function may temporarily deteriorate in the weeks following surgery but recovers over months to levels comparable with healthy population controls[247,250,251]. Allaix *et al.*[252] did a more detailed study, looking not just at quality of life but also ano-rectal manometry before and after TEM. Compared to pre-operative measures, manometry deteriorated initially but returned to baseline by 12 months.

A review of outcomes after CRT[253] found 13 articles published over the preceding three years and found, unsurprisingly, that the more aggressive the CRT the worse the functional outcome. The studies that included a healthy control group found similar outcomes except for diarrhoea and role

and social functioning, that were impaired. They also noted male sexual function was impaired by both surgery and CRT and noted that most studies could not asses female sexual function due to low response rates.

Several studies have looked at patients who have local excision after neoadjuvant radiation (RT) as part of an organ-preserving approach and have generally found little difference in the long term[254]. Coco *et al.*[255] compared RT+TEM with TEM alone in 47 patients and found no significant difference in ano-rectal function at one year. The CARTS study[256] assessed QLQ-30 up to three years, and LARS at least four years after treatment in 43 patients who had CRT then either TEM or TME. They noted slightly better outcomes in the TEM group, although 50% of patients reported major LARS in the long-term. Pucciarelli *et al.*[257] also noted slightly better bowel function in those who had local excision rather than TME after CRT at a median of 49 months.

Very few studies have directly compared RT alone with RT then local excision. A study of 100 'watch-and-wait' patients with near-complete clinical response[258] included 15 patients who had subsequent TEM for residual abnormality. At least three years after treatment, 22 RT alone and 7 RT+TEM patients completed a Vaizey questionnaire; the RT alone group reported good functional outcomes while outcomes were moderate for the RT+TEM group and 43% reported major incontinence.

No studies were found that report functional outcomes for patients having TEM then radiotherapy. Anecdotally, the outcomes locally were perceived as good, but some evidence is needed in order to inform patients embarking on this pathway.

7.1.5 Aims

1) To establish a local rectal cancer biobank

2) To investigate outcomes for patients undergoing neoadjuvant CRT for locally advanced rectal cancer, including rates of rectal preservation

3) To determine whether a gene signature of ECM genes predicts response to radiotherapy

4) To compare functional outcomes for patients after different rectal-preserving treatments for rectal cancer.

7.2 Methods

The methodology is detailed in Chapter 2. Patients participating in the Rectal cancer biobank who had neoadjuvant CRT following biopsy were identified from the database and the tissue samples retrieved.

7.2.1 Response to radiation

Response to radiation was determined by response on MRI scan six weeks after completion of CRT unless otherwise indicated, as this value was available for all patients. For patients who subsequently had surgical resection, histopathological assessment of response was also available. The gradings used are shown in Table 7.3; both are based on the proportion of tumour that has been replaced by fibrosis.

TABLE 7.2: TUMOUR RESPONSE GRADING, RADIOLOGICAL AND HISTOPATHOLOGICAL

Radiological grading		Histopathological grading	
mrTRG1	tumour not identified	**RG-1**	absence of residual cancer
mrTRG2	>75% fibrosis	**RG-2**	single cells or scattered small groups of cancer cells throughout fibrosis
mrTRG3	50% tumour/ fibrosis	**RG-3**	residual cancer outgrown by fibrosis
mrTRG4	>50% tumour, <25% fibrosis	**RG-4**	extensive residual tumour
mrTRG5	tumour signal only, no fibrosis	**RG-5**	absence of regressive changes

For those who radical surgery, the NAR (neoadjuvant rectal) score is also provided[259]. This is calculated as $[5\ ypN - 3\ (mrT - ypT) + 12]^2 / 9.61$.

7.2.2 Quality of life questionnaire

Patients attending the endoscopy department for surveillance following rectal-preserving treatment for rectal cancer between April 2017 and April 2019 were asked to complete a questionnaire at their follow-up visit. The investigator approached all eligible patients attending on one day of the week and collected the completed questionnaire before the patient left the department. Patients had completed treatment within the previous five years and at least eight weeks had passed since completing the last treatment, be this surgery or radiotherapy. All were disease-free at the time of evaluation. Patients who had undergone radical surgery were not included. The questionnaires were collected at a range of time points following treatment determined by logistical considerations. This approach was adopted because an earlier attempt at a more structured postal survey had a very low response rate.

7.3 Results

7.3.1 Rectal cancer biobank recruitment

Over a 2-year period, tissue biopsies and blood samples were collected from 98 patients with suspected rectal cancer; nine of these were in fact benign disease and so excluded. Of the remaining 89, 21 had already had treatment with radiotherapy and two had prior chemotherapy. Of the 66 that had not had prior radiation and/ or chemotherapy, 27 (41%) proceeded directly to radical TME surgery, without neoadjuvant treatment, 10 (15%) underwent local excision by TEM, 22 (33%) proceeded to radiotherapy and seven (11%) went initially for chemotherapy; of these, five subsequently had radiotherapy. The flow diagram for these patients is shown in Figure 7.1.

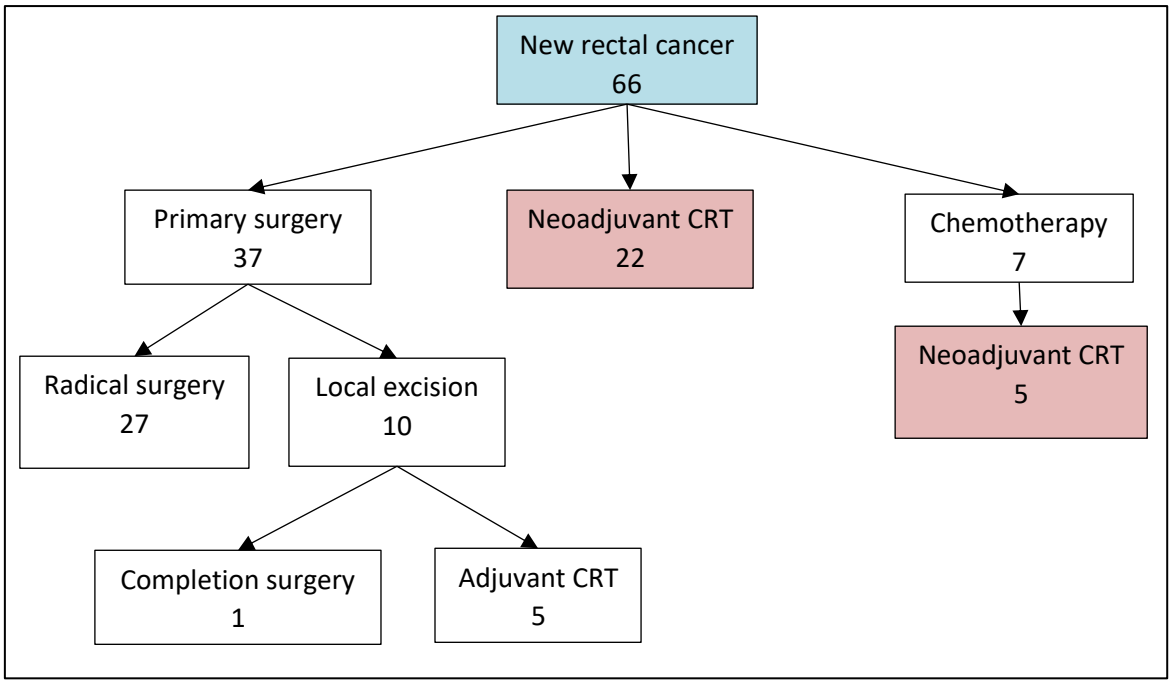


FIGURE 7.1: FLOW DIAGRAM FOR PATIENTS WITH NEWLY DIAGNOSED RECTAL CANCER RECRUITED INTO STUDY

The two patients who received chemotherapy alone were both female with median age 58. One achieved a complete clinical response and proceeded to radical surgery which did not reveal any residual tumour. The second had liver metastases at the time of diagnosis and demonstrated no response to multiple courses of chemotherapy, with continued growth of local and distant disease at last follow-up, 12 months after diagnosis.

The 27 undergoing radical surgery comprised 17 males and 10 females with median age 64 (range 37-82); on average this group was five years younger than the remainder. The operations involved 22 anterior resections, one TaTME and four APRs.

7.3.1.1 Local excision

The ten patients undergoing TEM comprised nine males and one female. The median age was 69 (range 47-81). Five underwent subsequent adjuvant CRT for adverse histopathological features; all these were disease-free at a median 22 months (range 8-31) following TEM. A further two were

considered for CRT but one refused, and one was not fit. One patient underwent a completion radical procedure six months after TEM and was well at last follow-up 25 months after TEM. Four patients commenced a surveillance regime following TEM; of these, three are currently disease-free at median 28 months from TEM. One elderly patient developed local recurrence and underwent a short course of radiotherapy one year after TEM; five months later the recurrence persists.

7.3.2 Chemoradiation

There were 27 patients who received neoadjuvant CRT, five of these additionally received chemotherapy with Oxaliplatin prior to CRT. This group comprised 17 males and 10 females with median age 69 (range 37-87). The tumours were in the lower third of the rectum in 70% (19/27). Two-thirds of patients received 52 Gy in 25 fractions (18/27); five received 45 Gy in 25 fractions and one received 50.4 Gy in 28 fractions. Two patients received a total pelvic dose of 76 Gy in 37 fractions as combined treatment for concomitant rectal and prostate cancers, and one patient received short-course radiation (25 Gy in 5 fractions) due to being unfit for CRT.

At assessment six weeks following completion of CRT, 10 achieved complete clinical response with no residual tumour on endoscopy and tumour regression grade 1 (mrTRG1) on MRI (complete radiological regression), seven achieved mrTRG2, with >75% fibrosis replacing the tumour, two showed mrTRG3, with 50% each tumour and fibrosis, two showed mrTRG4, with >50% tumour remaining and <25% fibrosis, and five showed mrTRG5 (no response to treatment).

For the five patients who received additional chemotherapy prior to CRT, one achieved complete response and two were mrTRG2 at the 6-week MRI scan, while two had no response to CRT. Figure 7.2 shows the outcomes for the different initial response groups.

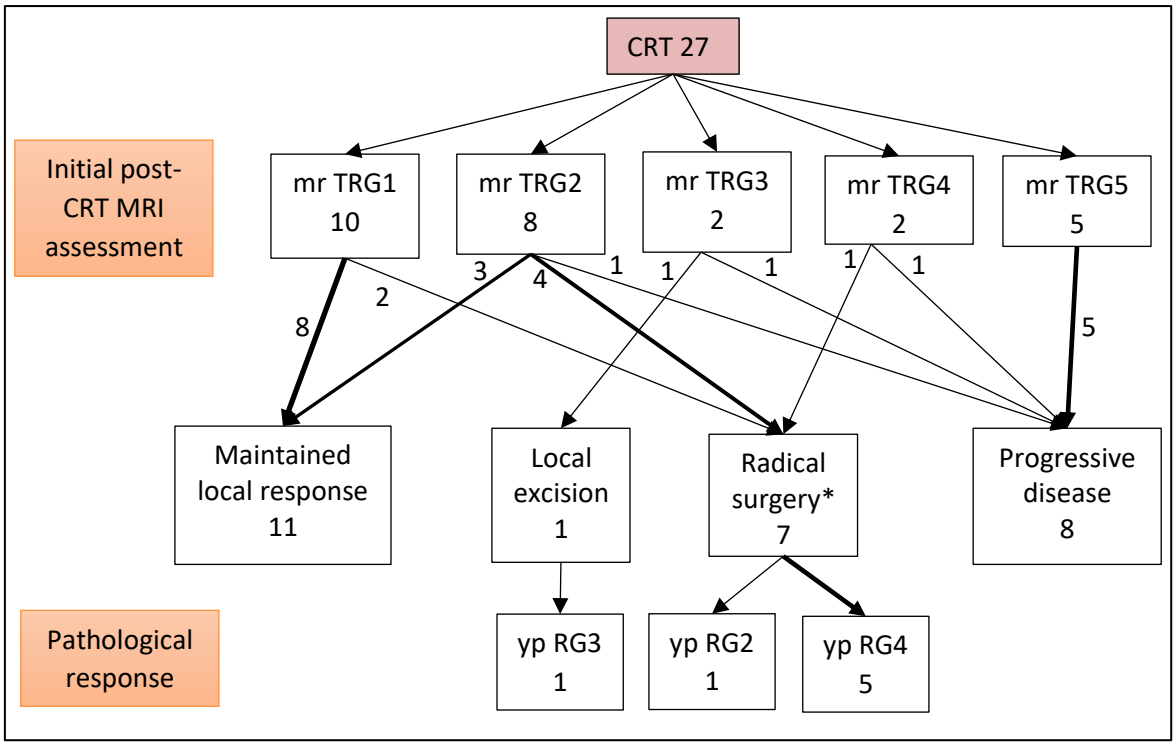


FIGURE 7.2: RESPONSE TO NEOADJUVANT CRT FOR 27 PATIENTS WITH RECTAL CANCER. THE INITIAL MRTRG AT 6 WEEKS IS SHOWN FOR ALL PATIENTS AND OUTCOME AT LAST FOLLOW-UP. YPTRG IS ALSO SHOWN FOR THOSE WHO HAD RESECTION. *ONE PATHOLOGY RESULT NOT YET AVAILABLE.

7.3.2.1 Early response to CRT

Of the 10 who achieved complete clinical response (mrTRG1), eight are continuing under surveillance on the 'watch-and-wait' pathway at a median of 19 months (range 6-24) following completion of CRT. One developed regrowth after 4 months so proceeded to radical surgery. Another, who was relatively young and remained in clinical complete response also underwent radical surgery 4 months after completing CRT; histopathology showed a ypT3 tumour, RG2 (scattered residual cancer cells).

Of the eight who initially achieved mrTRG2, four subsequently achieved complete clinical response: two remain on the 'watch-and-wait' pathway, at a median 17 months following CRT. Another developed regrowth after 12 months and is awaiting radical surgery. The fourth died of cardiac problems 4 months after CRT. Three underwent radical surgery at a median of 7 months after CRT;

these were younger than the overall group, with average age 60. The last one was lost to follow up for a period and presented with a perforated tumour 21 months following CRT; this patient has now died of disease.

For the two initial mrTRG3 tumours, one underwent local excision of the residual tumour by TEM 9 months after CRT. The other had progressive disease and died 4 months after CRT.

Both the two initial mrTRG4 tumours had chemotherapy after CRT. One underwent radical surgery 9 months after CRT and the other is awaiting surgery.

Of the five with no response to radiation, one subsequently developed some regression and had further chemotherapy and resection of liver metastases, however disease persists. The other four had progressive disease. Of the five, two died of disease at a median 8 months after CRT and three remain under palliative care at a median 17 months after completing CRT.

7.3.2.2 Pathological response

Seven (26%) patients have so far undergone surgery after CRT, with histopathology available to compare pathological response with early outcome. These seven include three males and four females with median age 59 (range 50-71). Surgery was undertaken at a median of 7 months (range 3-9) following completion of CRT. Figure 7.3 depicts the change in tumour staging, from the pre-treatment stage based on MRI scan to the final pathological stage. Also indicated is the response to CRT according to MRI scan six weeks after completion of CRT and the pathological regression grade. Most of the tumours were staged as T3 on MRI before treatment and as T3 on the histopathology of the resected tumour. There is no clear relationship between the early TRG noted on MRI six weeks after CRT and the RG noted on the resection specimen which is maybe not surprising given the passage of time between the two assessments.

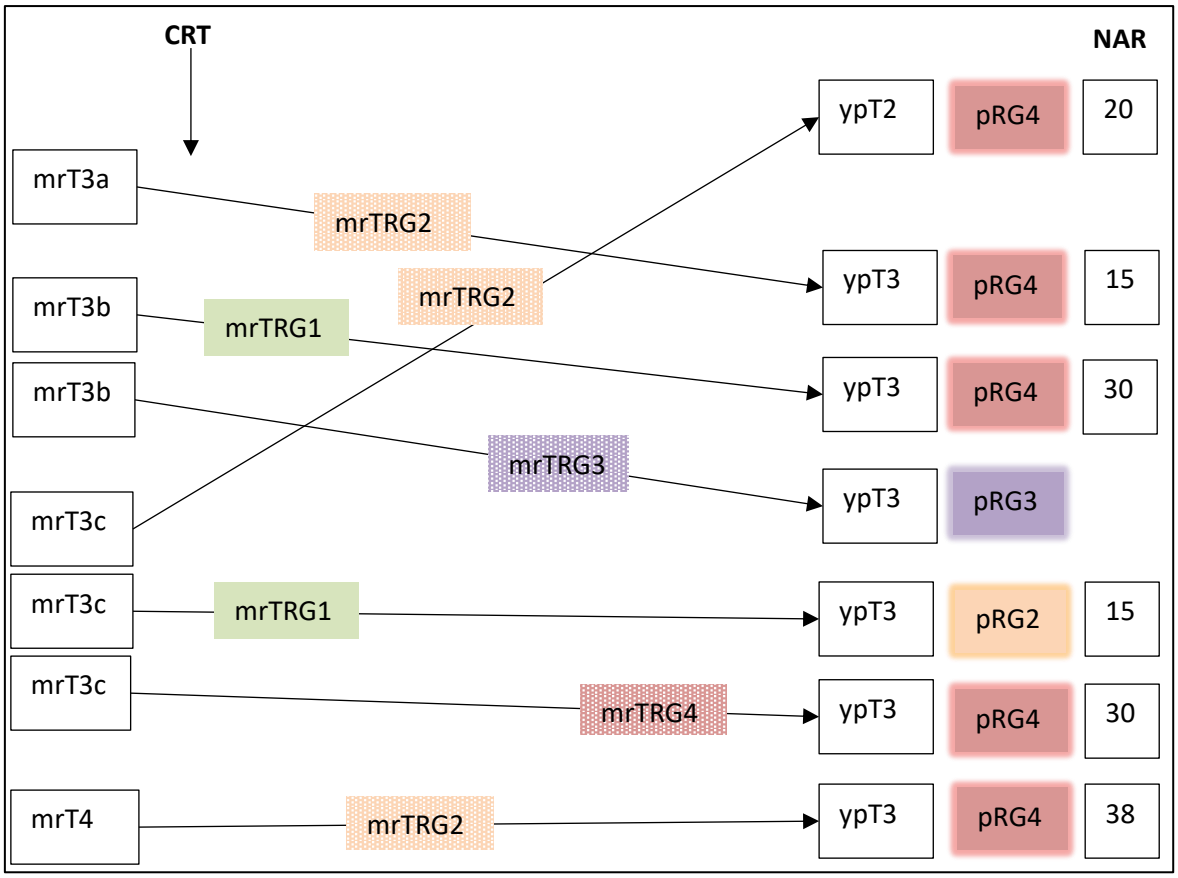


FIGURE 7.3: PROGRESSION OF TUMOUR RESPONSE TO CRT FOR SEVEN RESECTED TUMOURS. THE INITIAL MRI STAGE IS SHOWN, WITH MRTRG AT 6 WEEKS POST-CRT AND PROGRESSION TO FINAL HISTOPATHOLOGY RESULT, INDICATING TUMOUR STAGE, PATHOLOGIAL TUMOUR REGRESSION GRADE (PRG) AND NAR (NEOADJUVANT RECTAL SCORE) FOR RADICAL RESECTIONS.

The nodal status changed variably: of three staged as N0 on the initial MRI scan, one remained so and two were yN1c with collections of tumours cells in the mesorectum outside nodes on histopathology; of two initially staged as N1, one remained so and one was yN0; and similarly for two initially staged N2, one remained so and one was yN0. The NAR score, shown on Figure 7.3, is based on both ypN stage and change in T stage from mrT to ypT.

In most patients the presence of vascular invasion did not change over time; of four staged as V1, three remained so and one was yV0, while for three staged as V0, two remained so and one was yV1. On the staging scan the CRM was threatened in two and involved in five; on histopathology the resection margin was clear on six, and one, staged as involved, was R1.

7.3.2.3 Distant metastases

Ten patients (37%) have been diagnosed with distant metastases; in at least three, metastases were present at the time of diagnosis of rectal cancer. Two patients have had liver resections and one has had stereotactic ablative radiation to paraaortic nodal disease. All have had additional chemotherapy except two elderly patients who were not fit and so managed palliatively.

7.3.2.4 Outcomes

The median follow-up period was 15 months (range 1-28). At last follow up 11 patients (41%) had preservation of rectal function, with no significant local disease, 10 following CRT alone and one following CRT then local excision, however three had signs of metastatic disease. These patients had not started their treatment pathway with the intention of organ preservation, but were entered into a 'watch-and-wait' pathway following an early positive response to neoadjuvant treatment; patient age and comorbidity as well as choice also contribute to management decisions. Figure 7.4A shows the survival curve for preservation of a healthy functioning rectum. At 12 months the estimated rate of healthy rectal preservation is 56% (95% CI: 40-78).

Overall, at last follow-up, five patients had died of disease and one died of other causes. Nine patients are alive with disease and 12 are alive with no evidence of disease. Of the 11 surviving patients without rectal preservation, six have had radical surgery and two have had stomas for advanced local disease. One is awaiting radical surgery for re-growth 12 months after initial complete clinical response, and two are under palliative management. Figure 7.4B shows overall survival following CRT. The overall estimated survival at 12 months is 85% (95% CI: 72-100) and at 24 months is 79% (95% CI: 64-98).

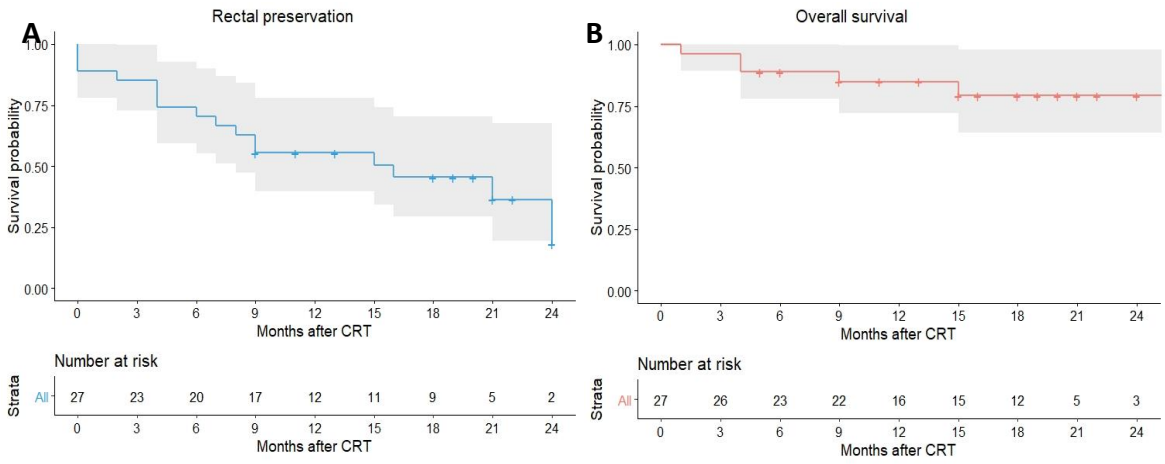


FIGURE 7.4: SURVIVAL CURVES AFTER NEOADJUVANT CRT INDICATING 95% CONFIDENCE INTERVAL. A. SURVIVAL WITH A HEALTHY FUNCTIONING RECTUM. **B.** OVERALL SURVIVAL.

7.3.3 Clinical markers

Regression analysis was used to assess any association between response to radiation and clinical factors including age, sex, tumour position and size and tumour characteristics on the staging MRI. The only significant predictor of a complete response (mrTRG1) on the early MRI scan was tumour stage on the staging MRI. A logistic regression model showed that patients with tumours staged as mrT3-4 were 11-times more likely not to achieve a complete response, compared to those staged with earlier tumours (log odds 2.39, 95% CI 0.66 – 4.4, p=0.01). On a linear regression model for tumour regression grade, again only tumour stage was significant.

There was a significant association between the early mrTRG and outcome, as shown in Figure 7.5 (χ^2 test, p = 0.009). Here, outcomes are divided into three groups: sustained complete, or near-complete response; some response but then regrowth or recurrence requiring treatment; and no response/ poor outcome.

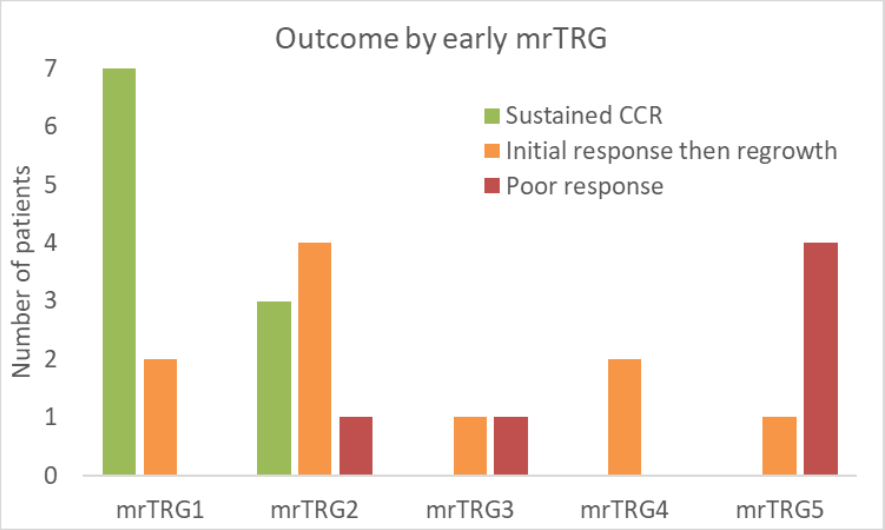


FIGURE 7.5: OUTCOME ACCORDING TO TRG STATUS AT 6-WEEK POST-CRT MRI SCAN, WITH OUTCOME DIVIDED INTO SUSTAINED (NEAR-)COMPLETE RESPONSE, INITIAL RESPONSE BUT THEN REGROWTH, AND NO OR POOR TUMOUR RESPONSE.

7.3.4 Molecular markers

The facilities where it was planned to process the samples became unavailable for continuing use during the study, so it has not yet been possible to obtain full results for the collected samples. Gene expression data for four genes: *COL5A1*, *COL5A2*, *COL11A1* and *FBN1*, was available for 14 of the patients. This subgroup comprised 10 males and four females, with median age 70 (range 37-87). Figure 7.6 shows the pre-treatment tumour stage, based on MRI scan, the TRG at the 6-week post-CRT MRI and the outcome for this subgroup. Three of these patients have had surgical resection.

The four analysed genes comprise three fibrillar collagen forming subunits and one which forms fibrillin. The scatter plots in Figure 7.7A show the correlation between the levels of these genes in the 14 samples.

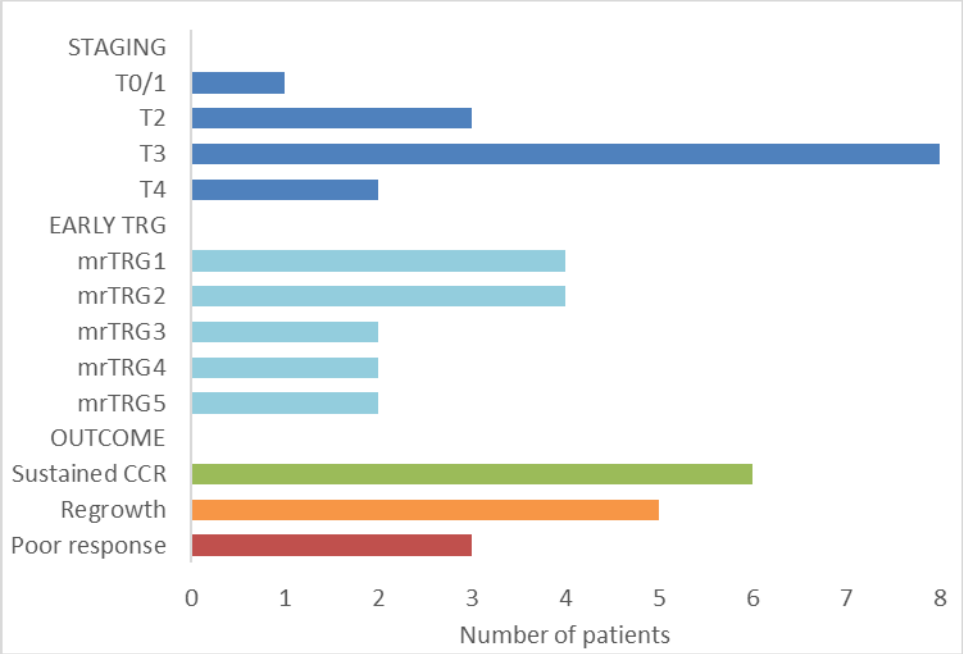


FIGURE 7.6: CHARACTERISTICS OF THE 14 PATIENTS WITH GENE EXPRESSION DATA AVAILABLE, SHOWING TUMOUR STAGE, TUMOUR REGRESSION GRADE ON 6-WEEK MRI AND OUTCOME.

For analysis, the logarithm of the gene expression values was used. Figure 7.7B shows the range of gene expression values according to early response to CRT. This shows a trend for higher levels of *COL5A1* and *FBN1* in the less responsive tumours on the 6-week MRI scan, while there is not much difference in levels of *COL5A2* or *COL11A1*.

Figure 7.7C shows the gene expression according to outcome. While this shows a trend for lower gene expression levels in those with a sustained complete response there are insufficient samples to give a definitive picture.

Gene expression values were compared with early tumour MRI regression grade using ordinal regression and logistic regression using a binary response for mrTRG1&2 vs mrTGR3-5. Similarly, gene expression was compared with outcome, both using an ordinal regression with three categories, and logistic regression for the binary value complete response or not. With the limited number of samples and genes tested, no significant associations were detected.

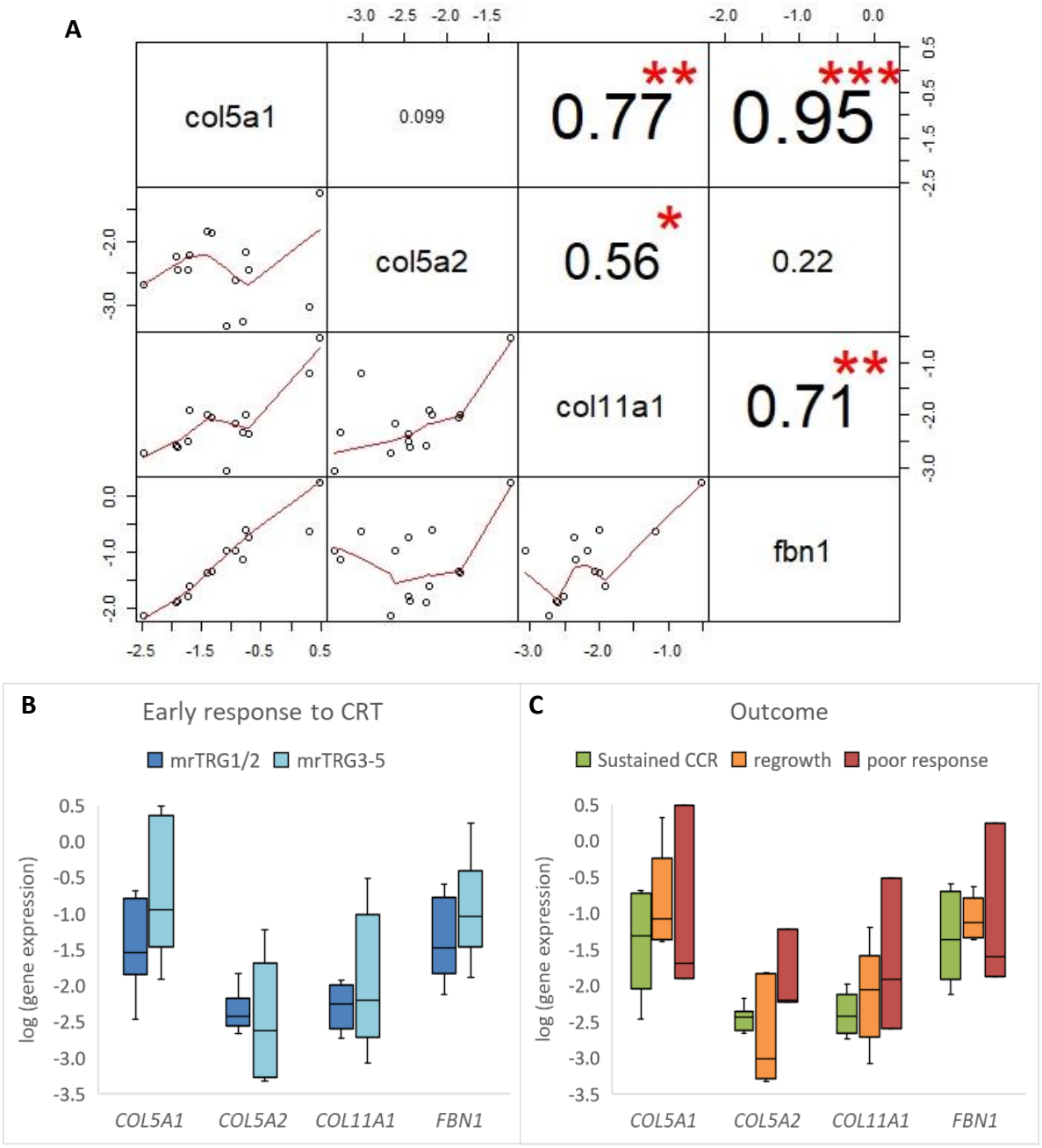


FIGURE 7.7: GENE EXPRESSION DATA FOR FOUR ECM GENES IN 14 RECTAL CANCERS TREATED WITH NEOADJUVANT CRT. A. CHART SHOWING CORRELATION PLOTS FOR LOGS OF GENE ENRICHMENT WITH A FITTED LINE IN THE LOWER PANEL AND PEARSON CORRELATION COEFFICIENT IN THE UPPER PANEL, WITH FONT SIZE INDICATING DEGREE OF CORRELATION AND RED ASTERISKS INDICATING SIGNIFICANCE LEVEL (***= P<0.001 ,**= P< 0.01 ,*= P<0.05, .= P<0.1, ' '=P>0.1). CHART PRODUCED USING PERFORMANCEANALYTICS R PACKAGE[260].**B.** EXPRESSION BY EARLY RESPONSE TO CRT AS ASSESSED AT 6-WEEK MRI. **C.** EXPRESSION BY OUTCOME DIVIDED INTO SUSTAINED (NEAR-)COMPLETE RESPONSE, INITIAL RESPONSE BUT THEN REGROWTH, AND NO OR POOR TUMOUR RESPONSE.

7.3.5 Quality of life after rectal-preserving treatments

Quality of life and functional questionnaires were completed by 120 of 127 (94%) approached patients who had rectal-preserving treatment of rectal cancer. These included 45 patients managed by radiotherapy (RT) followed by 'watch-and-wait', eight patients treated by RT followed by TEM, 49 patients managed by TEM alone and 18 patients managed by TEM followed by adjuvant RT because of high-risk histopathological features. Because of small numbers in the groups having both RT and TEM, in either order, these were combined into a single 'composite treatment' group. Two patients had stomas (one in TEM and one in RT group). Most patients fully completed the questionnaire. Some patients missed segments, most commonly the sexual function items, and occasionally single items were missed.

The median time between treatment and questionnaire completion was 7 months (interquartile range 4 – 26 months). Table 7.2 shows the demographic details. The groups were similar in terms of age, sex and follow-up period.

TABLE 7.2: DEMOGRAPHICS AND GLOBAL SCORES FOR PATIENTS UNDERGOING DIFFERENT RECTAL-PRESERVING TREATMENTS FOR RECTAL CANCER, SHOWING MEDIAN AND INTER-QUARTILE RANGE UNLESS OTHERWISE INDICATED

	RT alone n=45	**TEM alone n=49**	**Composite n=26**	**p value[1]**
Male n (%)	32 (71%)	28 (57%)	19 (73%)	0.25
Age	74 (65-80)	73 (65-80)	69.5 (66-77)	0.46
Time till questionnaire, months	7 (4-12)	6 (3-26)	19 (8-34)	0.083
EQ overall quality of life measures				
EQ-5D index, mean (std)	0.82 (0.25)	0.87 (0.14)	0.90 (0.15)	0.38
EQ VAS, mean (std)	77 (16)	83 (12)	79 (14)	0.11

[1] Kruskal-Wallis or χ^2 test

Table 7.2 also shows the EQ-5D results by group. The EQ-5D index was similar between the groups with an average above 0.8 in all groups and an average VAS over 75, indicating fairly good overall quality of life. For the VAS there was a non-significant trend for higher sores in the TEM only group. Tables 7.3 and 7.4 present the results for the QLQ-C30 and QLQ-CR29 questionnaires and Figure

7.8 shows the functional scales for each. There were no significant differences in the functional scales nor in the global health status score and QLQ-C30 summary score.

TABLE 7.3: QLQ-30 RESPONSES BY TREATMENT GROUP, SHOWING MEAN AND STANDARD DEVIATION

	RT alone n=45	TEM alone n=49	Composite n=26	p value[1]
Physical functioning	84.4 (22.0)	87.6 (14.4)	87.2 (15.3)	0.99
Role functioning	81.5 (27.1)	88.5 (19.2)	87.8 (18.0)	0.56
Emotional functioning	81.7 (23.1)	85.2 (18.8)	88.1 (16.5)	0.54
Cognitive functioning	85.6 (20.9)	91.7 (12.4)	90.4 (15.0)	0.36
Social functioning	81.9 (25.6)	89.2 (20.2)	86.5 (18.9)	0.30
Symptom scores				
Fatigue	22.0 (26.5)	16.2 (18.0)	20.5 (20.0)	0.65
Nausea/ vomiting	7.0 (16.9)	3.8 (15.5)	0.0 (0.0)	0.0061*
Pain	15.9 (27.7)	7.6 (15.4)	9.0 (17.8)	0.31
Dyspnoea	11.9 (25.8)	11.1 (18.6)	12.8 (21.2)	0.77
Insomnia	25.2 (33.5)	22.2 (24.1)	21.8 (24.8)	0.99
Appetite loss	11.9 (25.8)	6.2 (19.0)	7.7 (19.6)	0.44
Constipation	12.6 (24.9)	9.7 (21.7)	7.7 (14.3)	0.79
Diarrhoea	10.4 (21.1)	5.6 (15.9)	12.8 (23.2)	0.28
Financial impact	5.2 (18.7)	3.5 (10.3)	2.6 (9.1)	0.93
Overall scores				
Global health status	79.2 (16.5)	77.4 (18.0)	76.6 (19.9)	0.85
QLQ-Q30 summary score	84.5 (18.3)	89.2 (12.0)	88.3 (10.6)	0.78

[1] Kruskal-Wallis test

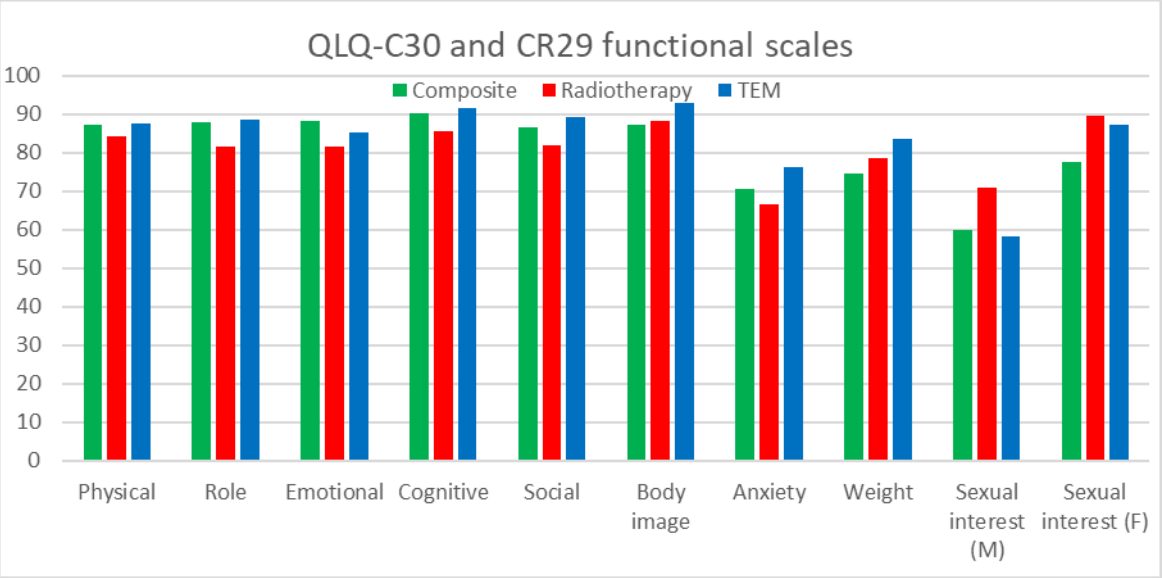


FIGURE 7.8: EORTC QLQ-C30 AND CR29 FUNCTIONAL SCALES, SHOWING MEAN SCORE FOR EACH TREATMENT GROUP.

TABLE 7.4: QLQ-CR29 RESPONSES BY TREATMENT GROUP, SHOWING MEAN AND STANDARD DEVIATION

	RT alone n=45	TEM alone n=49	Composite n=26	P value[1]
Body image	88.1 (20.1)	93.0 (10.3)	87.3 (14.2)	0.31
Anxiety	66.7 (30.5)	76.2 (24.5)	70.5 (25.5)	0.31
Weight	78.8 (32.2)	83.7 (26.5)	74.6 (31.5)	0.49
Sexual interest (Male)	70.8 (32.5) n=32	58.3 (34.7) n=28	60.0 (33.8) n=15	0.29
Sexual interest (Female)	89.7 (28.5) n=13	87.3 (32.4) n=21	77.8 (27.2) n=6	0.25
Symptom scores				
Urinary frequency	30.7 (23.0)	33.3 (25.7)	33.3 (23.6)	0.79
Urinary incontinence	14.4 (24.3)	11.6 (19.9)	12.8 (19.0)	0.91
Dysuria	1.5 (7.0)	3.4 (10.2)	2.6 (9.1)	0.59
Blood/ mucus in stools	10.6 (15.3)	2.4 (6.8)	17.9 (16.9)	0.000007*
Stool frequency	12.4 (13.1)	10.9 (14.1)	20.3 (19.4)	0.13
Flatulence	24.8 (25.0)	26.1 (26.2)	27.5 (25.9)	0.92
Faecal incontinence	12.8 (22.4)	7.2 (15.6)	17.4 (22.2)	0.10
Sore perianal skin	18.8 (26.3)	10.1 (20.9)	18.8 (24.3)	0.13
Embarrassment due to bowels	11.1 (17.7)	4.3 (11.4)	17.4 (22.2)	0.013*
Abdominal pain	5.3 (16.0)	6.1 (14.7)	1.3 (6.5)	0.29
Buttock pain	9.1 (16.6)	8.8 (17.7)	10.3 (18.3)	0.92
Bloating	11.4 (21.5)	10.9 (19.7)	12.8 (21.2)	0.90
Dry mouth	16.7 (26.4)	14.3 (19.2)	16.7 (25.4)	0.99
Hair loss	6.8 (15.4)	0.7 (4.8)	2.6 (9.1)	0.026*
Taste	12.9 (28.0)	4.1 (17.5)	7.7 (21.7)	0.080
Impotence	41.1 (38.8), n=30	45.2 (41.8), n=28	61.9 (36.6), n=14	0.25
Dyspareunia	6.7 (14.1), n=10	3.7 (10.8), n=18	5.6 (13.6), n=6	0.26

[1] Kruskal-Wallis test

7.3.5.1 Ano-rectal function

The QLQ-C30 includes symptom scores for constipation and diarrhoea and the QLQ-CR29 includes six bowel-related symptom scores. There were significant differences between groups for blood and mucus in the stool and embarrassment due to bowel function, with the most symptoms in the composite group and the fewest in the TEM only group. Figure 7.9A shows a radar chart of median level of bowel symptoms.

LARS and Vaisey scores are shown in Table 7.5. Both the LARS and Vaizey scores were lowest (better) in the TEM group and highest in the composite groups, although this was only significant

for LARS (Figure 7.9B). The mean LARS score for the TEM only group was 17, in the 'no LARS' range, while for all other groups the mean score was in the 'minor LARS' range. Figure 7.9C shows the LARS severity grouping for each treatment while Figure 7.9D shows the results of ANCOVA demonstrating the LARS score increased with age in each group.

TABLE 7.5: ANO-RECTAL, URINARY AND SEXUAL FUNCTION AFTER DIFFERENT RECTAL-PRESERVING TREATMENTS FOR RECTAL CANCER, SHOWING MEAN AND STANDARD DEVIATION UNLESS OTHERWISE INDICATED.

	RT alone n=45	TEM alone n=49	Composite n=26	p value[1]
Ano-rectal function				
LARS score	20.8 (12.6)	17.0 (12.5)	25.1 (11.2)	0.032*
Vaizey score	5.3 (5.4)	4.5 (4.6)	7.4 (5.9)	0.16
Urinary function				
IPSS score	5.9 (5.4)	6.0 (4.6)	7.4 (5.9)	0.56
IPSS QOL score	1.2 (1.0)	1.6 (1.4)	1.8 (1.4)	0.14
Sexual function: male				
Sexually active? n (%)	7/30 (23.3%)	14/25 (56.0%)	4/12 (33.3%)	0.040*
IIEF-5 score **Sexually active only**	15.4 (7.0) 19.8 (4.4)	15.7 (8.3) 19.9 (5.9)	13.6 (8.5) 20.7 (4.0)	0.054
Sexual function: female				
Sexually active? n (%)	1/12 (8.3%)	4/17 (23.5%)	1/5 (20.0%)	0.49
MFSQ global score	29.3 (14.2), n=3	26.6 (7.4), n=5	28.0 (n/a), n=1	0.28

[1] Kruskall-Wallis or χ^2test

7.3.5.2 Urinary and sexual function

The QLQ-CR29 includes three urinary-related symptom scores (Table 7.4). There were no significant differences between groups for urinary frequency, incontinence, or dysuria. The IPSS scores are shown in Table 7.5. The majority of patients in all groups were mildly symptomatic according to the IPSS score, and were delighted, pleased or mostly satisfied with their quality of life due to urinary symptoms, with no significant differences between groups (Figure 7.10A).

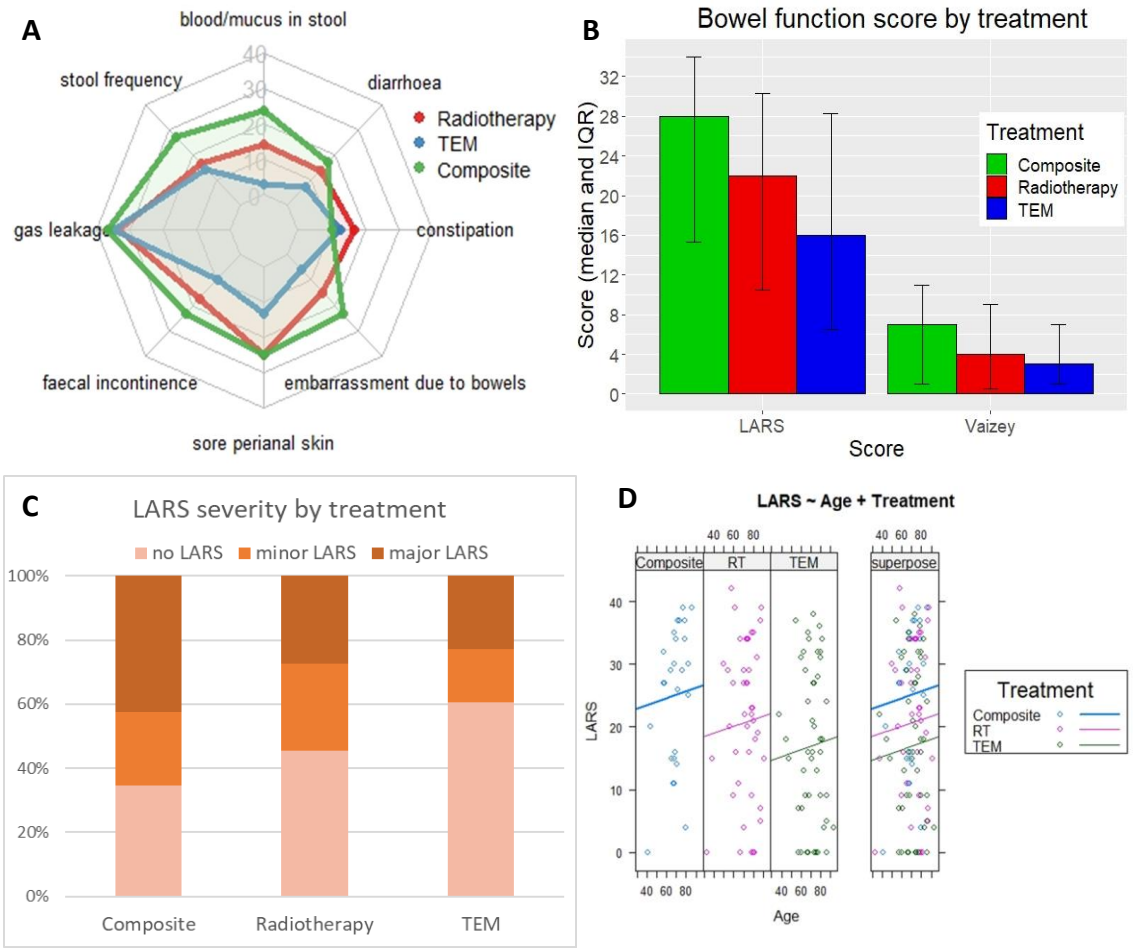


FIGURE 7.9: BOWEL SYMPTOMS REPORTED BY EACH TREATMENT GROUP. A. RADAR PLOT SHOWING MEAN SYMPTOM SCORE FOR QLQ-C30 AND QLQ-CR29 BOWEL SYMPTOMS FOR EACH GROUP. **B.** LARS AND VAISEY SCORES SHOWING MEDIAN AND IQR. **C.** LARS SCORE SEVERITY IN EACH TREATMENT GROUP. **D**. ANCOVA ANALYSIS SHOWING LARS SCORE INCREASING WITH AGE WITHIN EACH TREATMENT GROUP.

Only 31% (31/101) patients indicated that they were sexually active; the median age for these patients was 65, compared with 72 overall, and the rate was higher for men (37%) than women (18%). Although the questionnaire did not ask for further details, many patients volunteered that the reason was age or lack of spouse/ partner.

The QLQ-CR29 (Table 7.4) includes questions on interest in sex, impotence, and dyspareunia. Most patients indicated a moderate interest in sex. There was a moderate level of impotence in all groups, while dyspareunia was rarely reported. There were no significant differences between groups.

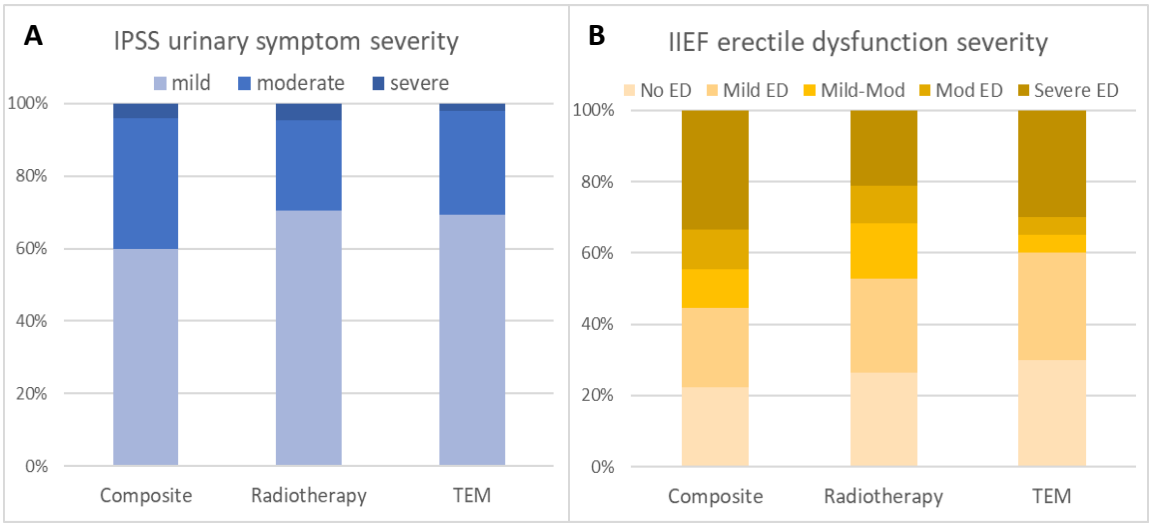


FIGURE 7.10: URINARY AND SEXUAL FUNCTION REPORTED BY EACH TREATMENT GROUP. A. IPSS URINARY SYMPTOM SEVERITY. **B.** IIEF SEVERITY OF ERECTILE DYSFUNCTION.

The IIEF questionnaire was completed by 48 of 79 men (61%) (Table 7.5). There were no significant differences between groups (Figure 7.10B).

Only 9 of 41 women (22%) fully completed the MFSQ questionnaire (Table 7.5). These showed a moderate level of sexual function, with no difference between groups.

7.4 Discussion

One major achievement of this project was establishing a local biobank for tissue and blood samples from rectal cancer patients. While it has still not been possible to analyse sufficient samples to provide definitive results about potential molecular predictors of responsiveness to radiotherapy, processes have been developed for collecting and storing tissue which will enable future work.

The study recruited 66 patients with newly diagnosed rectal cancer over a two-year period. This is not a comprehensive picture of all new rectal cancer patients, as patients were recruited

opportunistically when attending for endoscopy on a certain day of the week, when medical and research nursing staff were available. Nonetheless it does provide a snapshot of patient management. Just under half (41%) the patients proceeded directly to radical surgery. Ten patients (15%) embarked on an organ-preserving pathway involving primary local excision and nine have preserved rectal function. The other 44% of patients had neoadjuvant treatment. Over the two years only six of these (22%) have proceeded to radical surgery, with another two awaiting surgery. A few had aggressive disease which progressed despite radiation and chemotherapy, while 41% (11/27) have a sustained complete, or near-complete clinical local response to treatment and are continuing an organ-preserving pathway.

All patients with a sustained response showed TRG 1-2 on the MRI scan six weeks following completion of CRT. All those with early TRG 3-5 had progressive or recurrent disease, as did half of those with TRG2. The literature indicates a longer interval since CRT increases the odds of a complete pathological response[261] but the present results show that incomplete regression on the MRI scan six weeks after CRT makes a significant response unlikely.

The poor outcomes for the five patients who demonstrated no response to CRT on the six-week MRI, three of whom developed metastases in the interval between the initial staging scan and the scan six weeks after completing CRT, are consistent with the concern raised by Poynter *et al.*[241] that for this group, that perforce remains unidentified at the time of commencing neoadjuvant treatment, CRT may worsen prognosis by delaying a potentially effective treatment.

7.4.1 mrTRG as an indicator of response

The mrTRG on the early post-CRT MRI scan was used to assess response to radiation, since this was available for all patients, whereas few had pathological data on response available. Although numbers are small, our results show the six-week mrTRG to be a useful marker which significantly correlates with outcome. The TRIGGER trial[262] is currently assessing the value of mrTRG as a

biomarker to stratify patients according to response to CRT. In this trial patients with mrTRG 1-2 on the six-week post-CRT scan receive chemotherapy and then undergo surveillance, while those with mrTRG 3-5 receive chemotherapy then surgery if there is still not a good response.

Sclafani *et al.*[263] have shown that mrTRG does not agree well with pathological regression grade, and that a combination of mrTRG with pRG provides a more accurate predictor of survival. The pRG is not strongly associated with tumour stage. Mancini *et al.*[264] demonstrated a spread in pRG for both low and high pathological tumour stage. This is explained by the distribution of residual cancer cells through the rectal wall after CRT. Duldulao *et al.*[265] examined 111 specimens and found that the distribution of residual cancer cells correlated with the pre-treatment T stage, with surviving cells located preferentially at the invasive front of the tumour.

Evidence that the mrTRG is an effective prognostic determinant can allow more confident decision making about continuing an organ-preserving treatment pathway, even in the absence of confirmation from a pathological specimen.

7.4.2 Quality of life after rectal-preserving treatment

Most patients reported a fairly good quality of life after each of the organ-preserving treatments, indicating that the treatment modalities are equivalent in terms of impact on quality of life, apart from some areas of ano-rectal function. The TEM group reported lower LARS scores and fewer symptoms of blood and mucus and embarrassment due to bowel function, while the composite group had more symptoms. Due to small numbers in the composite groups, especially the RT+TEM group, the investigators' prior belief that radiotherapy after TEM was more benign than radiotherapy before TEM could not been proved. While there is less operative morbidity with this sequence, as noted in Chapter 4, the longer-term effects on bowel function are not negligible. Chapter 4 has shown that adjuvant radiation reduces the risk of local recurrence in those with high-risk tumours, and the MDT survey shows this is being practised in several centres around the UK

even though strong evidence so far is lacking. A trial is awaited, and more rigorous assessment of these functional outcomes will be crucial part of this trial, to obtain the evidence needed to fully inform clinicians and patients about the balance of risks and benefits.

Limitations of this study are the small numbers in some groups, the lack of baseline comparison questionnaires and variable follow-up, occasioned by the pragmatic nature of the study. Nevertheless, it was felt that given the paucity of existing evidence on this topic, it was worthwhile proceeding to analyse the available data.

Any rectal-preserving strategy commits the patient to a prolonged period of intense surveillance so any local recurrence can be promptly detected and treated. This surveillance in itself may be a source of anxiety to patients. To address this, more detailed quantitative studies are required including in-depth interviews with patients to fully explore the decision-making process, any decision-regret experienced, and how the information provided to patients can be optimised to aid this process, at the very difficult time, shortly after the diagnosis of rectal cancer, when patients need to make difficult decisions about their management. These aspects will be incorporated into the upcoming Vorates trial.

7.4.3 Future directions

The biobank established as the initial step in this study will allow the investigation into biomarkers for radiosensitivity or radioresistance to continue. Another feature of the biobank that has not yet been utilised is the serial collection of blood samples for all patients, including those who have developed recurrence. There is increasing evidence that biomarkers derived from the rectal tumour and present in the blood may provide evidence about response to treatment and an early indicator of recurrence. Tie *et al.*[266] analysed serial blood samples from 159 patients with locally advanced rectal cancer who underwent CRT then radical surgery. They detected circulating tumour DNA (ctDNA) in 77% of samples prior to treatment. Only 8% still had detectable ctDNA 4-6 weeks after

CRT and 12% had detectable levels after TME surgery. There was a trend for less complete pathological response in those with detectable ctDNA after CRT, but numbers were small, and this was not significant. They suggest that the lack of strong correlation indicates that ctDNA analysis within a short interval of completing CRT cannot distinguish between minimal and no residual disease. On the other hand, ctDNA detectable after TME surgery was strongly prognostic. Patients with ctDNA detectable after surgery had an estimated 3-year recurrence-free survival of only 33% compared with 87% in those without detectable ctDNA. The presence of detectable ctDNA prior to treatment did not carry prognostic value. They hypothesise that ctDNA present at diagnosis derives, in the vast majority of patients, from the primary tumour. Once the primary tumour is removed, detectable ctDNA indicates (micro-)metastatic disease.

Another potential biomarker present in the blood is extracellular vesicles (EV). These are small membranous vesicle secreted by cancer cells which contain various molecules including proteins and microRNA. There are several types, most of which bud off from the cell membrane, but one type, exosomes, are the end-product of recycled endosomes and originate from inward budding of the cell membrane[267]. It has been proposed that these exosomes will come to play a role in monitoring colorectal cancer progression and making treatment decision[267]. Ostenfeld *et al.*[268] focussed on microRNA from EVs. They found certain microRNAs were elevated in the presence of colorectal cancer, some of which significantly reduced after tumour removal, suggesting potential application for both screening and recurrence detection.

These novel techniques are still in their infancy but will undoubtedly come to play a role in the diagnosis and monitoring of rectal cancer. The samples stored in the biobank established here will enable ongoing investigation into such markers in this patient population, and will contribute to optimising the management pathway for patients who choose a rectal preserving approach.

Chapter 8: Conclusion

8.1 Main findings

8.1.1 Sentinel lymph node assessment

The Sentirect trial aimed to demonstrate the feasibility of a magnetic nanoparticle system to identify sentinel lymph nodes in early rectal cancer. So far, the trial has demonstrated that it is technically feasible to use the system and has made progress towards optimising dosage and timings. It has not yet demonstrated that the technique can reliably identify sentinel nodes which would permit lymph node biopsy during local excision. This technique does not yet have a role in managing early rectal cancer, but merits continued investigation to ensure it is not rejected prematurely because of still-developing experience, as it would provide very valuable prognostic information if the node could be reliably identified and excised.

Even if the technique cannot be shown to safely enable lymph node biopsy, nanoparticle injection may be worth further investigation in a radiology trial, if it can be shown to improve the diagnostic accuracy of MRI scan in detecting lymph node involvement in 'borderline' nodes in early rectal cancer.

8.1.2 TEM database analysis

TEM database analysis allowed determination of recurrence rates for the three post-TEM management pathways and showed overall recurrence rates are lower than historic rates. There was a relatively high incidence of positive resection margins, generally considered a particularly high-risk feature, but this was compensated for by a structured yet flexible approach to post-TEM management with rigorous surveillance and fully informed discussion with the patient.

Outcomes for completion surgery are not good, although numbers were small, whereas adjuvant radiation provides a definite advantage compared with surveillance alone for higher risk tumours and extends the stage of tumour that can be comfortably managed with a rectal preserving

approach. Furthermore, salvage surgery in the case of recurrence is not necessarily a bad option, if the recurrence is detected early, as it allows patients a longer period of rectal preservation and has similar survival outcomes compared to primary radical surgery when calculated from the time of initial treatment.

If the cancer has apparently been treated by complete polypectomy, it is not worth undertaking local excision of the area unless there is residual adenoma, however a careful endoscopic assessment is required to establish this. Similarly, if there has been an apparently complete response to neoadjuvant radiation, local excision is unlikely to add benefit and carries a risk of significant post-operative morbidity. On the other hand, if there is residual tumour, around half will develop recurrence within a year of local excision, so again it is worth careful consideration as to whether the risks of surgery are justified.

A few patients, despite optimal local treatment, whether completion surgery or complete polypectomy, will develop distant metastases, sometimes many years later. This indicates an aggressive tumour, but as yet no clear feature that predicts this aggressive behaviour, which would allow closer surveillance or more aggressive systemic treatment, has been identified.

The UK survey of early rectal cancer management showed a range of practice and strong support for a trial to further assess the value of adjuvant CRT. Some centres are uncomfortable with local excision as a treatment modality if the tumour is more advanced than T1sm1/2; such tumours only comprised a quarter of the present study. A more liberal usage of local excision in Oxford is enabled by appropriate choice of post-TEM management pathway, decided in consultation with the patient, who needs to be fully informed of the risks and benefits associated with the various options, and willing to comply with a rigorous surveillance regime if a pathway other than completion surgery is chosen. We also engaged with patients by undertaking a patient acceptability survey to assess their opinion on the Vorates trial feasibility, in particular randomisation to a radiotherapy arm. The majority of patients (10/12) presented with the questionnaire replied that they were willing to take

part in a study that meant they would be randomly allocated to have radiotherapy or not after a local excision of a rectal cancer.

8.1.3 Prognostic factors

A number of factors have been found of significance in this well-defined, homogenous population of early rectal cancers; these factors are not necessarily the same as found in broader studies of colorectal cancer, highlighting the importance of the present results to the management of this patient group.

Perineural invasion was identified as a more significant risk factor for recurrence than many of the other routinely reported features. Another readily available feature now is the absence of the three common mutations: *APC*, *TP53* and *KRAS*; which suggests a higher recurrence risk. This data has only recently become routinely available but should now be pursued further.

There was no value in MSI status, ESTIMATE stroma and immune scores, chromatin heterogeneity, ploidy or indicators of TSA polyp origin or Wnt pathway ligand dependency, all of which in less selective settings have shown some predictive capability. Stratification using the CMS groupings was of minimal value. The CRIS grouping, based on epithelial genes was of no value, confirming the importance of the stromal contribution is this set of early cancers.

The high prognostic value of the ratio of desmoplastic to inflamed stroma is a very promising finding, as it is a fairly quick and cheap technique (once the AI algorithm has been developed) and is superior to conventional stroma fraction measurement. This could be readily employed in routine clinical practice to guide decisions about post-TEM management.

Gene signatures indicating higher recurrence risk and poor responsiveness to radiotherapy are promising but further work is needed before they can find practical application in everyday clinical management.

8.1.4 Adenoma-carcinoma progression

Many of the early cancers in this series were caught in the transition from adenoma to carcinoma, allowing insights into this process. ECM remodelling is the critical difference between the two portions of the tumour. It seems likely that switching on these genes in the cancer cells is a very early step in the malignant transformation in rectal cancer. The remodelling then stiffens the ECM and induces EMT which converts some epithelial cells into CAFs which perpetuate the growth by further remodelling the ECM and distorting the basement membrane, allowing invasion and metastasis. The CAFs also express *IGF2* which also promotes ECM remodelling and invasion. This insight into these critical early steps raises the possibility of a therapeutic target.

8.1.5 Radiotherapy sensitivity

A biomarker or gene signature to indicate tumour resistance to radiotherapy is worth pursing further, so patients with resistant tumours could be spared exposure to ineffective radiation. This needs further exploration. For patients having neoadjuvant radiation, mrTRG on the scan six weeks later provides a useful prognostic marker of disease progression that can be used to modify the treatment strategy.

8.2 Summary

This thesis set out to investigate potential prognostic markers that could be applied in clinical practice to better inform the rectal preserving approach for managing early rectal cancer. It has demonstrated that local excision, combined with appropriate post-TEM management, can be safely applied to a range of cancer stages.

Although rectal preserving treatment has been proved safe and effective for many patients with early rectal cancer, only a minority of patients are treated, or even offered treatment, in this way. The national survey showed that there is a persisting reluctance to consider local excision, even with adjuvant radiation, as safe or effective for anything more than very early tumours. Demonstrating good outcomes is important in changing opinion among clinicians managing rectal cancer, so that more patients may be offered the opportunity of rectal preservation.

Good outcomes depend on appropriate selection, which relies on an accurate assessment of prognosis for an individual patient, and meticulous follow-up, with early intervention in case of any sign of recurrence. More accurate and individualised indicators of prognosis, such as the desmoplastic to inflamed stroma ratio or a specific gene signature, mean that patients can receive a more detailed picture of the outcomes to be expected with the various treatment pathways before reaching a decision.

Much work remains to be done, as identified in the individual chapter discussions, to continue to take this forward, particularly with a structured clinical trial to assess the value of adjuvant radiotherapy, analysis of the stored biobank blood samples for early markers of recurrence, and a qualitative study to gain a greater appreciation of patients' understanding of the risks and how best to support their decision making process. The new knowledge gained from this future work will further enhance the rectal preserving management of patients with early rectal cancer.

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Appendix

Appendix 1: Publications based on this work

1. Jones HJS, Al-Najami I, Cunningham C. Quality of life after rectal-preserving treatment of rectal cancer, *Eur J Surg Oncol*. Accepted 15/7/2020, DOI: 10.1016/j.ejso.2020.07.018
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This work also contributed to the following publication

1. Kleeman SO, Koelzer VH, Jones HJ, et al. Exploiting differential Wnt target gene expression to generate a molecular biomarker for colorectal cancer stratification. *Gut*, 2020; 69(6): 1092-1103, DOI: 10.1136/gutjnl-2019-319126.

Appendix 2: Early rectal cancer questionnaire

Early rectal cancer questionnaire

1. Does your unit offer local excision for early rectal cancer (ERC)?
   - ☐ Yes
   - ☐ No

2. If so, what techniques do you offer?
   - ☐ TEMS
   - ☐ TAMIS
   - ☐ TEO
   - ☐ Flexible endoscopic resection
   - ☐ Parks' per-anal excision
   - ☐ Refer to another centre

Other – please specify

3. Does your MDT manage early rectal cancer?
   - ☐ Yes, we manage all cases of ERC in our MDT
   - ☐ Yes, we manage most cases and only refer select cases to another MDT
   - ☐ No, we refer most or all cases of ERC to another MDT

Other – please specify

4. If so, what options are open to patients in your unit after local excision of early rectal cancer?
   - ☐ Surveillance
   - ☐ (Chemo)- radiotherapy
   - ☐ Contact radiotherapy or brachytherapy
   - ☐ Completion radical surgery
   - ☐ Refer to another centre

Other – please specify

5. According to your MDT, which if any of the following factors would mandate completion major resection in a patient who is fit for major surgery?
   - ☐ Poor tumour differentiation
   - ☐ Lymphatic invasion
   - ☐ Vascular invasion
   - ☐ Tumour budding
   - ☐ Peri-neural invasion
   - ☐ Cancer cells at resection margin
   - ☐ Depth of invasion into the submucosa (see below for details)
   - ☐ Width of invasive front (see below for details)

Other – please specify

6. If depth of invasion mandates major surgery which cut-off would you apply?

- Sm1
- Sm2
- Sm3
- 500 micrometres
- 1000 micrometres
- 2000 micrometres

Other – please specify

7. If width of invasive front mandates major surgery, which cut-off would you apply?

- 1 mm
- 5 mm
- 10 mm
- 15 mm

Other – please specify

8. If a patient who is fit for major surgery had any of the above "mandatory" features but did not want completion major surgery and fully understood the risks and implications of this decision, would you offer?

- Surveillance and no additional treatment
- (Chemo)-radio therapy and surveillance
- Refer to another centre

Other – please specify

9. If a patient who is unfit or high risk for major surgery had these "mandatory" features would you offer:

- Surveillance and no additional treatment
- (Chemo)-radio therapy and surveillance
- Refer to another centre

Other – please specify

10. Would you consider taking part in a clinical trial randomising patients after local excision of early rectal cancer between surveillance alone, versus adjuvant (chemo)-radiotherapy and surveillance?

- Yes
- No

Other – please specify

11. Any other comments?

Appendix 3: Sentirect trial documents

A3.1 Consent form

REC Reference: _____

Participant Identification Number for this trial: _____

CONSENT FORM

Title of Study: Feasibility of sentinel lymph node biopsy in rectal cancer (SentiRect)

Researcher: Mr Chris Cunningham

Please initial box

1. I confirm that I have read the information sheet dated 15 March 2019 (version 1.3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

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

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

4. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers, including at commercial organisations. ☐

5. I agree to routine video recording of my operation. ☐

6. I agree to my General Practitioner being informed of my participation in the study. ☐

7. I understand that all data will be stored securely and is covered by the Data Protection Act 1998. ☐

8. I agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

A3.2 Patient information sheet for Group A (ex-vivo group)

Churchill Hospital
Old Road
Headington
Oxford
OX3 7LE
Tel: 01865 741 841

Feasibility of sentinel lymph node biopsy in rectal cancer (SentiRect)

PATIENT INFORMATION SHEET – STUDY GROUP A

Please take your time to think about whether you would like to take part in the SentiRect study. More details are provided below and your medical team will be happy to answer any questions.

Invitation to take part in the SentiRect research study (Group A)

We are inviting you to take part in a research study that is investigating a technique to take a lymph node biopsy in people with rectal cancer. Before you decide whether or not you wish to take part in this study, you need to understand why the research is being done and what it what it would involve for you.

Please take your time to think about whether you would like to take part in this study or not. Talk to other people about the study if you want, and ask us if there is anything that is not clear, or if you would like more information.

What is the purpose of the study?

The purpose of the study is to see whether magnetic nanoparticles can help to find lymph nodes which may be involved in rectal cancer.

Why are lymph nodes important in rectal cancer?

Rectal cancer, like many cancers, can spread to nearby lymph nodes. Lymph nodes act as a filter in the body so cancer cells that spread away from the tumour often go first to a lymph node – in fact the first lymph node they go to is called the “sentinel” lymph node. For rectal cancer these lymph nodes are located in the fatty tissue around the rectum. One of the reasons for doing MRI and ultrasound scans before surgery is to see whether the lymph

nodes are involved, but unfortunately these scans are not 100% reliable for determining whether there are any cancer cells in the lymph nodes.

What are the benefits of examining the sentinel lymph node?

If we can identify the sentinel lymph node and remove it, the pathologist can examine it to see if it contains any cancer cells. If there are no cancer cells in the sentinel lymph node, this generally means that the cancer has not spread away from the main tumour so a less radical operation should be sufficient to remove the cancer. However if there are cancer cells in the sentinel lymph node, this indicates that that the tumour has started to spread and a more radical operation will generally be required to deal with the cancer. This method of looking at the lymph nodes is more reliable than scans. So knowing about the sentinel lymph node allows us to make better decisions about which type of operation is most suitable.

What is the SentiRect technique?

This technique uses a non-toxic tracer material called Magtrace to identify the sentinel lymph node. This tracer consists of tiny nanoparticles of iron which can be magnetised. When these particles get into the body's tissue, they are filtered out and removed from the system by the lymph nodes. The particles are so small that they then stay trapped in the lymph node. We can then use a hand-held probe called Sentimag which generates a small magnetic field. This magnetises the iron nanoparticles so the probe can then locate the sentinel lymph node. This technique does not involve any radioactivity.

Has this technique been used in other cancers?

In breast cancer this technique is commonly used to identify and remove the breast's sentinel lymph node in the armpit. This means that the patient and medical staff can make a more informed decision about the most appropriate operation to treat the cancer. There isn't yet a really effective way to identify the sentinel node in the tissue around the rectum in patients with rectal cancer. This study will use the same technique used in breast cancer to see if it works in rectal cancer.

How will the SentiRect technique be used?

During endoscopy, the tracer will be injected into the lining of the bowel around the tumour, about a week before surgery. Following this an MRI scan will be done to see where the tracer has gone, either later that day, or on the day of surgery. During surgery we will also inject some green dye called ICG (indocyanine green) that is often used to identify lymph nodes. After surgery we will use the Sentimag probe to look for the sentinel lymph node in the segment of bowel and surrounding tissue that has been removed. We will also look for

the ICG using a near infrared imaging system to give us the best chance of successfully finding the lymph nodes. The specimen will then go to the pathologist as normal.

How does the study work?

This study is in two groups, depending on the type of operation you are scheduled to have.

For group A, we are inviting people who are having major surgery to remove the tumour and the tissue around the rectum which contains lymph nodes. After surgery we will look at the tissue that has been removed and perform some tests on it. The tissue will also be analysed in the standard way by a pathologist. This group will involve 20 people with rectal cancer. We will look to see whether the tracer method, that we already know works in breast cancer, also works for finding the sentinel lymph node in rectal cancer. We will also look to see whether the injection causes any side effects or makes surgery more difficult.

For the other group, B, we are inviting people who are having a TEM (transanal endoscopic microsurgery) procedure to remove their tumour. During the operation, after the tumour has been removed, we will use the probe to find the lymph node and remove it. The lymph node, along with the tumour, will be analysed by a pathologist in the standard way.

Why have you been invited to take part?

All your endoscopy, scan and biopsy results have been reviewed in the Colorectal Cancer Multidisciplinary Team (MDT) meeting. Your surgeon and a number of other specialist doctors and nurses have discussed your case to determine what is the most appropriate course of treatment for you. The type of surgery recommended for you means you would be eligible to take part in this study. Taking part in the study will not affect the type of surgery you have or your routine clinical care.

Do I have to take part?

No – taking part in research is always voluntary. If you decide not to take part, you don't have to give a reason. If you decide to take part you will be asked to sign a consent form at your next visit, after you have had time to think about it. Even if you agree, you are still free to withdraw at any time without giving a reason. Your doctor may also at any time decide it is in your best interest to withdraw you from the study. Whatever you decide, the treatment for your rectal cancer will not be affected.

What would the study involve for you?

What will happen to me if I decide to take part in the SentiRect study (Group A)?

The treatment you receive will be the same as you would have received even if you were not in the study, but there will be an extra injection of the Magtrace tracer during your endoscopy and an extra MRI scan after your endoscopy. There will also be an extra injection of ICG into the tumour during your surgery.

You will come to the endoscopy department for a flexible sigmoidoscopy or colonoscopy about a week before your operation is due for your surgeon to assess your tumour. At this time you will be asked to sign the consent form if you want to take part in the study, and if you agree the tracer will be injected around the tumour during the endoscopy. You will not be able to feel this but may watch it on the TV screen if you wish. After the endoscopy you will remain in the recovery room for about 1 hour so we can check that there aren't any side effects from the injection. You will then go to the Radiology department for an MRI scan, either later that day or on the day of surgery, before your operation. This purpose of this is to see where the injected tracer has gone, and see if we can identify the sentinel lymph node.

About a week later you will come into hospital for your operation. Being in the study will not affect the operation apart from the injection of ICG dye into the tumour. The operation may be recorded on video as part of standard practice. After the operation we will examine the tissue that is removed with the Sentimag probe to look for lymph nodes, and also use a near infrared imaging system to look for the ICG dye. The specimen will then go to the pathologist who will examine the tumour as normal, but in addition they will look especially at the lymph nodes using the Sentimag probe. After surgery you will go to the ward for the normal post-operative care. Your involvement in the study will finish when you are discharged from hospital.

<u>What are the possible benefits from taking part in SentiRect?</u>

We cannot promise the study will help you, but your participation in the study will provide valuable information on the treatment of rectal cancer and this will be used for the benefit of future patients. When you come to your routine clinic visit after surgery we will be able to inform you whether the tracer was useful for locating your sentinel lymph node. We will also let you know the results of the standard pathology examination of the removed bowel and lymph nodes.

<u>What are the possible risks of taking part in SentiRect?</u>

There is a very small chance of an adverse allergic reaction to the injection, which may cause rash, itching, dizziness, lightheadedness, or very rarely, an anaphylactic reaction. These symptoms generally settle down without any treatment, but you will be given a phone

number to call in case you do develop any of these symptoms after leaving the hospital. People who have a known intolerance to iron or dextran compounds should not take part in this study. The injection may also cause some discolouration of the tissues around the rectum that the surgeon may notice during surgery. People with a known intolerance to iodine should not have the ICG injection during surgery as there is a small risk of an allergic reaction.

After your endoscopy you will have an extra MRI scan which may cause some inconvenience to you. There are no risks associated with an extra MRI scan.

What will happen if I don't want to carry on with the study?

You can decide not to continue with the study at any time, and this will have no effect on your medical care. If you do withdraw we will remove any identifiable data about you from the study data files. If you would like to withdraw from the study, you should contact Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

What if there is a problem?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study during the course of this study, you should contact Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

NHS bodies are legally liable for the negligent acts and omissions of their employees. If you are harmed whilst taking part in a clinical trial as a result of negligence on the part of a member of the trial team this liability cover would apply.

Non-negligent harm is not covered by the NHS indemnity scheme. The Oxford University NHS Trust, therefore, cannot agree in advance to pay compensation in these circumstances.

In exceptional circumstances an ex-gratia payment may be offered.

You will also be able to contact the Patient Advice and Liaison Service (PALS) in the first instance (01865 221473).

Will my taking part in the study be kept confidential?

- General Data Protection Regulations (GDPR) require mention of data controller (Oxford University Hospitals NHS Foundation Trust) and more explicit details about what personal data will be held by whom, for what purposes, and for how long. For example:

- We will be using information from you and your medical records in order to undertake this study. Research is a task that we perform in the public interest. Oxford University Hospitals NHS Foundation Trust, as sponsor, is the data controller. This means that we, as Oxford University Hospitals NHS Foundation Trust researchers, are responsible for looking after your information and using it properly. We will use the minimum personally-identifiable information possible. We will keep identifiable information about you for less than three months after the study has finished. We will store the anonymised research data and any research documents with personal information, such as consent forms, securely at the Oxford University Hospitals NHS Foundation Trust for ten years after the end of the study as part of the research record.

- The local study team may use your details, e.g. name, NHS number, home address, and contact details, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. They will keep identifiable information about you from this study for less than three months after the study has finished. Consent forms will be archived at site for 10 years after the study has finished.

- Your rights to access, change, or move your personal information may be limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. Further information about your rights with respect to your personal data is available at https://www.ouh.nhs.uk/privacy/default.aspx.

- You can find out more about how we use your information by contacting Mr Chris Cunningham (chriscunningham@nhs.net).

If you decide to take part, all information collected about you during the course of the study will be kept strictly confidential in the same way as all of your other medical records. Your study data will only be identified by initials and a participant ID number. Information will be collected on paper and then transferred to electronic form. Both paper and electronic data will be securely stored under the provisions of the 1998 Data Protection Act.

Your GP, and the other doctors involved in your clinical care, will be notified of your participation in the study.

Responsible members of the Oxford University Hospitals NHS Trust and hosting organisation may be given access to data for monitoring and/or audit of the study to ensure we are complying with regulations.

Anonymised data collected during the course of the study may be passed on to other organisations which may include commercial organisations.

What will happen to the results of the study?

Once the study is finished we will publish the results in a medical journal so that others can benefit. No individual patient will be identified in any publication. In line with clinical trial guidelines, at the end of the study the data will need to be securely archived for a minimum of 10 years. Arrangements for confidential destruction will then be made.

Will any expenses be paid?

Reasonable travel expenses for any visits additional to normal care will be reimbursed on production of receipts, or a mileage allowance provided.

Who is organising and funding the research?

This study is being sponsored by Oxford University Hospitals NHS Trust and has been developed by the surgeons at Oxford University Hospitals NHS Trust. It is funded by an Impact Acceleration Award which supports the development of healthcare technology, and Endomagnetics, the company that manufactures Magtrace and the Sentimag probe. The chief researcher is Mr Chris Cunningham.

Who has reviewed the study?

All research in the NHS in looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the Oxford Research Ethics Committee.

Where can I get further information?

If you have any further questions about this study please discuss them with your doctor, your specialist nurse or the SentiRect chief investigator: Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

Thank you for taking the time to consider this study.

A3.2 Patient information sheet for Group B (in-vivo group)

Churchill Hospital
Old Road
Headington
Oxford
OX3 7LE
Tel: 01865 741 841

Feasibility of sentinel lymph node biopsy in rectal cancer (SentiRect)

PATIENT INFORMATION SHEET – STUDY GROUP B

Please take your time to think about whether you would like to take part in the SentiRect study. More details are provided below and your medical team will be happy to answer any questions.

Invitation to take part in the SentiRect research study (Group B)

We are inviting you to take part in a research study that is investigating a technique to take a lymph node biopsy in people with rectal cancer. Before you decide whether or not you wish to take part in this study, you need to understand why the research is being done and what it what it would involve for you.

Please take your time to think about whether you would like to take part in this study or not. Talk to other people about the study if you want, and ask us if there is anything that is not clear, or if you would like more information.

What is the purpose of the study?

The purpose of the study is to see whether magnetic nanoparticles can help to find lymph nodes which may be involved in rectal cancer.

Why are lymph nodes important in rectal cancer?

Rectal cancer, like many cancers, can spread to nearby lymph nodes. Lymph nodes act as a filter in the body so cancer cells that spread away from the tumour often go first to a lymph node – in fact the first lymph node they go to is called the “sentinel” lymph node. For rectal cancer these lymph nodes are located in the fatty tissue around the rectum. One of the

reasons for doing MRI and ultrasound scans before surgery is to see whether the lymph nodes are involved, but unfortunately these scans are not 100% reliable for determining whether there are any cancer cells in the lymph nodes.

What are the benefits of examining the sentinel lymph node?

If we can identify the sentinel lymph node and remove it, the pathologist can examine it to see if it contains any cancer cells. If there are no cancer cells in the sentinel lymph node, this generally means that the cancer has not spread away from the main tumour so a less radical operation should be sufficient to remove the cancer. However if there are cancer cells in the sentinel lymph node, this indicates that that the tumour has started to spread and a more radical operation will generally be required to deal with the cancer. This method of looking at the lymph nodes is more reliable than scans. So knowing about the sentinel lymph node allows us to make better decisions about which type of operation is most suitable.

What is the SentiRect technique?

This technique uses a non-toxic tracer material called Magtrace to identify the sentinel lymph node. This tracer consists of tiny nanoparticles of iron which can be magnetised. When these particles get into the body's tissue, they are filtered out and removed from the system by the lymph nodes. The particles are so small that they then stay trapped in the lymph node. We can then use a hand-held probe called Sentimag which generates a small magnetic field. This magnetises the iron nanoparticles so the probe can then locate the sentinel lymph node. This technique does not involve any radioactivity.

Has this technique been used in other cancers?

In breast cancer this technique is commonly used to identify and remove the breast's sentinel lymph node in the armpit. This means that the patient and medical staff can make a more informed decision about the most appropriate operation to treat the cancer. There isn't yet a really effective way to identify the sentinel node in the tissue around the rectum in patients with rectal cancer. This study will use the same technique used in breast cancer to see if it works in rectal cancer.

How will the SentiRect technique be used?

During endoscopy, the tracer will be injected into the lining of the bowel around the tumour. Following this an MRI scan will be done to see where the tracer has gone, either later that day, or on the day of surgery. At surgery, about a week after the injection, we will use the Sentimag probe to look for the sentinel lymph node during surgery. The probe will be

inserted into the rectum and it will give a signal to show exactly where the magnetic tracer is. This lymph node will be removed and sent to the pathologist along with the tumour.

How does the study work?

This study is in two groups, depending on the type of operation you are scheduled to have.

For group A we are inviting people who are having major surgery to remove the tumour and the tissue around the rectum which contains lymph nodes. After surgery we will look at the tissue that has been removed and perform some tests on it. The tissue will also be analysed in the standard way by a pathologist.

For group B we are inviting people who are having a TEM (transanal endoscopic microsurgery) procedure to remove their tumour. During the operation, after the tumour has been removed, we will use the probe to find the lymph node and remove it. The lymph node, along with the tumour, will be analysed by a pathologist in the standard way. This group will involve 20 people with rectal cancer. We will look to see whether the tracer method, that we already know works in breast cancer, also works for finding the sentinel lymph node in rectal cancer. We will look to see whether the injection causes any side effects or makes surgery more difficult. We will also see how easy it is to remove the sentinel lymph node at surgery once we have identified it using the tracer.

Why have you been invited to take part?

All your endoscopy, scan and biopsy results have been reviewed in the Colorectal Cancer Multidisciplinary Team (MDT) meeting. Your surgeon and a number of other specialist doctors and nurses have discussed your case to determine what is the most appropriate course of treatment for you. The type of surgery recommended for you means you would be eligible to take part in this study. Taking part in the study will not affect the type of surgery you have or your routine clinical care

Do I have to take part?

No – taking part in research is always voluntary. If you decide not to take part, you don't have to give a reason. If you decide to take part you will be asked to sign a consent form at your next visit, after you have had time to think about it. Even if you agree, you are still free to withdraw at any time without giving a reason. Your doctor may also at any time decide it is in your best interest to withdraw you from the study. Whatever you decide, the treatment for your rectal cancer will not be affected.

What would the study involve for you?

What will happen to me if I decide to take part in the SentiRect study (Group B)?

The treatment you receive will be almost the same as you would have received even if you were not in the study, but there will be an extra injection of the Magtrace tracer during your endoscopy and an extra MRI scan after your endoscopy. There will also be an extra step in your surgery.

You will come to the endoscopy department for a flexible sigmoidoscopy or colonoscopy about a week before your operation is due for your surgeon to assess your tumour. At this time you will be asked to sign the consent form if you want to take part in the study, and if you agree the tracer will be injected around the tumour during the endoscopy. You will not be able to feel this but may watch it on the TV screen if you wish. After the endoscopy you will remain in the recovery room for about 1 hour so we can check that there aren't any side effects from the injection. You will then go to the Radiology department for an MRI scan, either later that day or on the day of surgery, before your operation. This purpose of this is to see where the injected tracer has gone, and see if we can identify the sentinel lymph node.

About a week later you will come into hospital for your transanal endoscopic microsurgery (TEM) operation. The first part of your operation will be the same as if you were not in the study. The operation may be recorded on video as part of standard practice. After the tumour has been removed there will be an extra step. While you are still under the anaesthetic we will use the Sentimag probe to locate the sentinel lymph node in the tissue around the rectum. If we can locate the node, we will then remove it during the same operation. After the operation the tumour and the lymph node will go to the pathologist who will examine it as normal.

After surgery you will go to the ward for the normal post-operative care. Your involvement in the study will finish when you are discharged from hospital.

What are the possible benefits from taking part in SentiRect?

We cannot promise the study will help you, but your participation in the study will provide valuable information on the treatment of rectal cancer and this will be used for the benefit of future patients. When you come to your routine clinic visit after surgery we will be able to inform you whether the tracer was useful for locating your sentinel lymph node and if it was, whether the sentinel lymph node contained any cancer cells.

What are the possible risks of taking part in SentiRect?

There is a very small chance of an adverse allergic reaction to the injection, which may cause rash, itching, dizziness, lightheadedness, or very rarely, an anaphylactic reaction. These symptoms generally settle down without any treatment, but you will be given a phone number to call in case you do develop any of these symptoms after leaving the hospital. People who have a known intolerance to iron or dextran compounds should not take part in this study. The injection may also cause some discolouration of the tissues around the rectum that the surgeon may notice during surgery.

After your endoscopy you will have an extra MRI scan which may cause some inconvenience to you. There are no risks associated with an extra MRI scan.

During your surgery, the extra step to locate and remove the sentinel lymph node could add some extra time to the operation and may slightly increase the risk of complications after the surgery such as bleeding.

What will happen if I don't want to carry on with the study?

You can decide not to continue with the study at any time, and this will have no effect on your medical care. If you do withdraw we will remove any identifiable data about you from the study data files. If you would like to withdraw from the study, you should contact Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

What if there is a problem?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study during the course of this study, you should contact Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

NHS bodies are legally liable for the negligent acts and omissions of their employees. If you are harmed whilst taking part in a clinical trial as a result of negligence on the part of a member of the trial team this liability cover would apply.

Non-negligent harm is not covered by the NHS indemnity scheme. The Oxford University NHS Trust, therefore, cannot agree in advance to pay compensation in these circumstances.

In exceptional circumstances an ex-gratia payment may be offered.

You will also be able to contact the Patient Advice and Liaison Service (PALS) in the first instance (01865 221473).

Will my taking part in the study be kept confidential?

- General Data Protection Regulations (GDPR) require mention of data controller (Oxford University Hospitals NHS Foundation Trust) and more explicit details about what personal data will be held by whom, for what purposes, and for how long. For example:

- We will be using information from you and your medical records in order to undertake this study. Research is a task that we perform in the public interest. Oxford University Hospitals NHS Foundation Trust, as sponsor, is the data controller. This means that we, as Oxford University Hospitals NHS Foundation Trust researchers, are responsible for looking after your information and using it properly. We will use the minimum personally-identifiable information possible. We will keep identifiable information about you for less than three months after the study has finished. We will store the anonymised research data and any research documents with personal information, such as consent forms, securely at the Oxford University Hospitals NHS Foundation Trust for ten years after the end of the study as part of the research record.

- The local study team may use your details, e.g. name, NHS number, home address, and contact details, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. They will keep identifiable information about you from this study for less than three months after the study has finished. Consent forms will be archived at site for 10 years after the study has finished.

- Your rights to access, change, or move your personal information may be limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. Further information about your rights with respect to your personal data is available at https://www.ouh.nhs.uk/privacy/default.aspx.

- You can find out more about how we use your information by contacting Prof Chris Cunningham (chriscunningham@nhs.net).

If you decide to take part, all information collected about you during the course of the study will be kept strictly confidential in the same way as all of your other medical records. Your study data will only be identified by initials and a participant ID number. Information will be collected on paper and then transferred to electronic form. Both paper and electronic data will be securely stored under the provisions of the 1998 Data Protection Act.

Your GP, and the other doctors involved in your clinical care, will be notified of your participation in the study.

Responsible members of the Oxford University Hospitals NHS Trust and hosting organisation may be given access to data for monitoring and/or audit of the study to ensure we are complying with regulations.

Anonymised data collected during the course of the study may be passed on to other organisations which may include commercial organisations.

What will happen to the results of the study?

Once the study is finished we will publish the results in a medical journal so that others can benefit. No individual patient will be identified in any publication. In line with clinical trial guidelines, at the end of the study the data will need to be securely archived for a minimum of 10 years. Arrangements for confidential destruction will then be made.

Will any expenses be paid?

Reasonable travel expenses for any visits additional to normal care will be reimbursed on production of receipts, or a mileage allowance provided.

Who is organising and funding the research?

This study is being sponsored by Oxford University Hospitals NHS Trust and has been developed by the surgeons at Oxford University Hospitals NHS Trust. It is funded by an Impact Acceleration Award which supports the development of healthcare technology, and Endomagnetics, the company that manufactures Magtrace and the Sentimag probe. The chief researcher is Mr Chris Cunningham.

Who has reviewed the study?

All research in the NHS in looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the Oxford Research Ethics Committee.

Where can I get further information?

If you have any further questions about this study please discuss them with your doctor, your specialist nurse or the SentiRect chief investigator: Mr Chris Cunningham (tel: 01865 235657, email: chriscunningham@nhs.net).

Thank you for taking the time to consider this study.

A3.4 GP information sheet

Churchill Hospital
Old Road
Headington
Oxford
OX3 7LE
Tel: 01865 741 841

Dear Dr …….

Name ……. DoB ……. NHS No ……

Your patient, named above, has recently been diagnosed with rectal cancer and is due for surgery shortly.

We are currently undertaking a study called SentiRect to assess the feasibility of sentinel lymph node biopsy in rectal cancer. Accurately assessing the stage of a patient's tumour prior to surgery is very important in deciding on the most appropriate operation. If lymph nodes are involved, radical surgery is generally required to reduce the risk of local recurrence, whereas for early tumours with no lymph node involvement less radical surgery with transanal endoscopic microsurgery (TEM) may be sufficient, and offers the patient much less morbidity than radical surgery.

Although various imaging techniques are available to assess the patient's mesorectal lymph nodes prior to surgery, these are not always accurate. Sentinel lymph node biopsy is commonly used in breast cancer patients. We are investigating whether a magnetic nanoparticle tracer (Magtrace) will enable us to identify and biopsy the sentinel lymph node in the mesorectum in rectal cancer patients.

Patients will receive a tracer injection around the tumour during their pre-operative endoscopy. They will have an MRI scan either on the day of injection or surgery. They will then follow Group A or Group B depending on their scheduled operation.

Group A patients will proceed to their scheduled radical surgery. The specimen will be investigated by magnetic probe in addition to the standard histopathology examination.

Group B patients will proceed to their scheduled TEM operation and during surgery a magnetic probe will be used to locate the sentinel node so it can be biopsied.

Patients' involvement in the study will end when they are discharged from hospital after their operation.

Your patient has kindly consented to participate in the SentiRect study: Group A ☐ Group B ☐

If you have any queries regarding your patient's management or if you require any further information about the study, please feel free to contact me. Please file this letter in the patient's notes.

Yours sincerely,

Chris Cunningham

Appendix 4: Model diagnostic plots

A4.1 Diagnostic plots for propensity score matching, Chapter 4, 4.3.5.2.

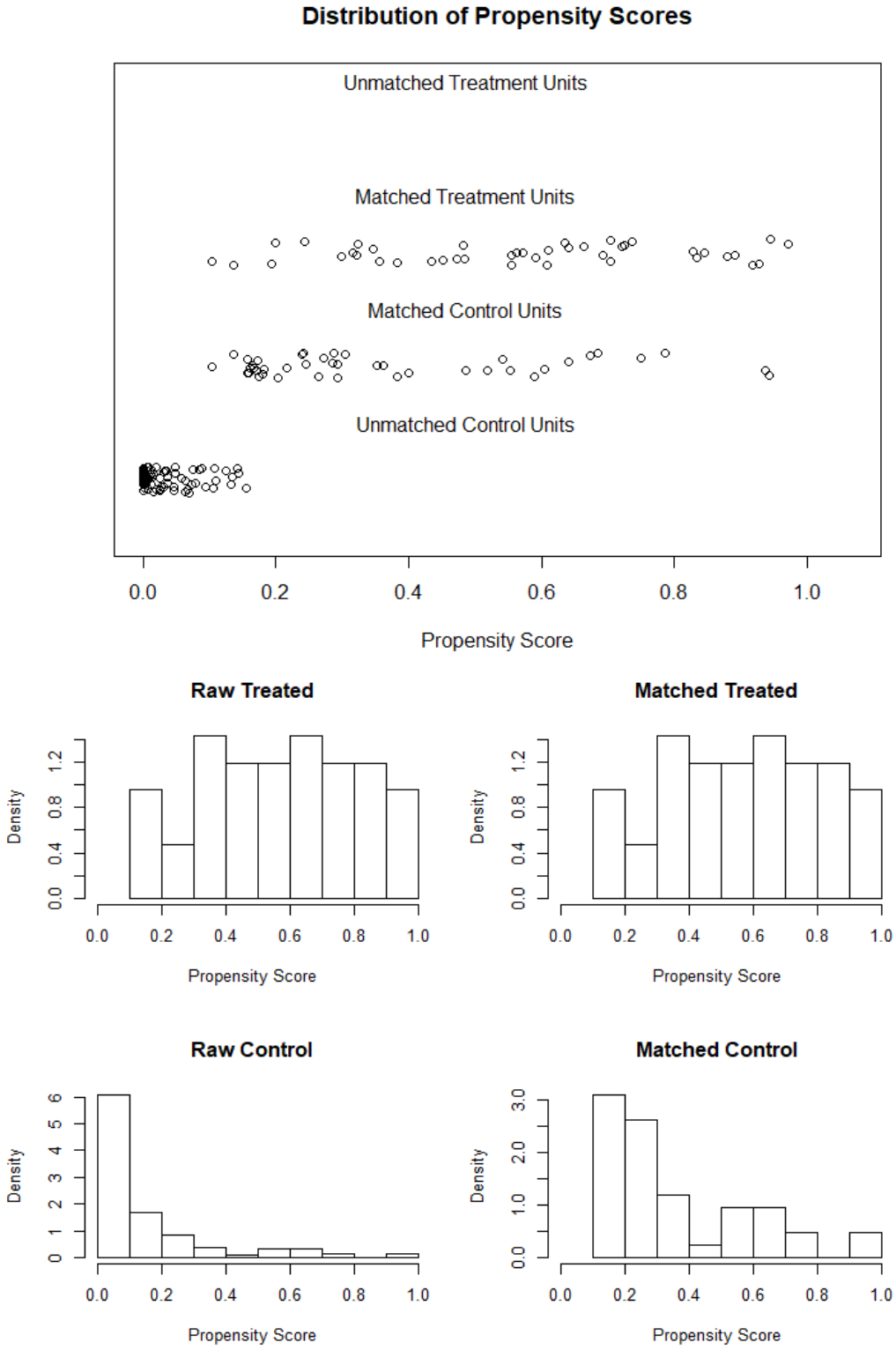


FIGURE A1: DIAGNOSTIC PLOTS FOR PROPENSITY SCORE MODEL. A. DISTRIBUTION OF PROPENSITY SCORES. **B.** HISTOGRAMS OF PROPENSITY SCORES FOR RADIOTHERAPY (=TREATED) AND SURVEILLANCE (=CONTROL) GROUPS. RAW INDICATES THE WHOLE GROUP AND MATCHED REFERS TO THE 42 MATCHED CASES.

A4.2 Model diagnostics for regression analysis Chapter 5, 5.3.1.6, poor prognosis

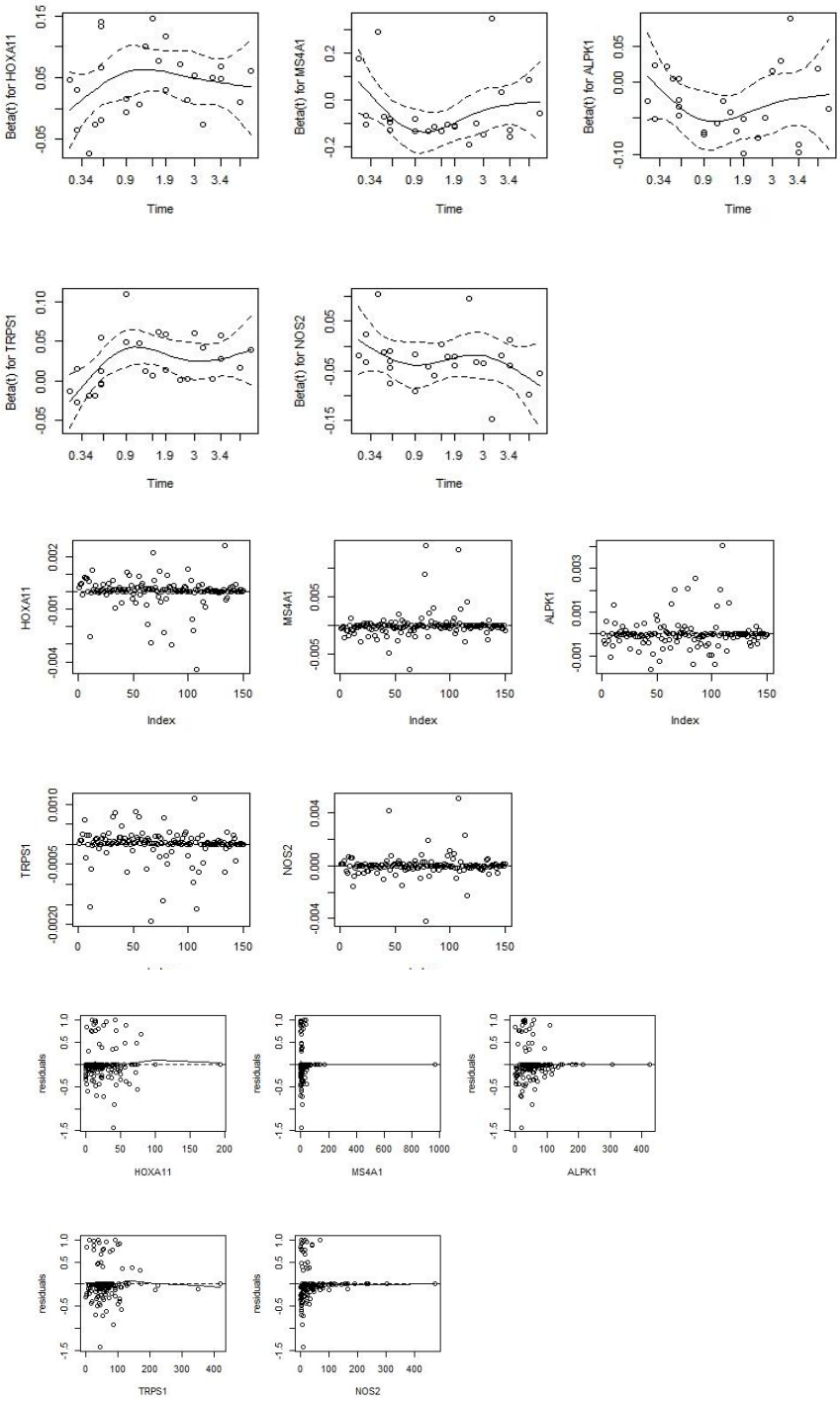


FIGURE A2: MODEL DIAGNOSTICS FOR POOR PROGNOSIS 5-GENE MODEL. A: SCHOENFELD RESIDUALS DO NOT SHOW ANY SYSTEMATIC TRENDS OVER TIME**. B:** INFLUENTIAL CASES PLOT, SHOWING THE ESTIMATED CHANGES IN THE REGRESSION COEFFICIENTS IF EACH CASE IN TURN WAS DELETED**. C:** MARTINGALE RESIDUALS TO ASSESS FOR NON-LINEARITY.

A4.3 Model diagnostics for regression analysis Chapter 5, 5.3.1.2, recurrence after adjuvant radiotherapy

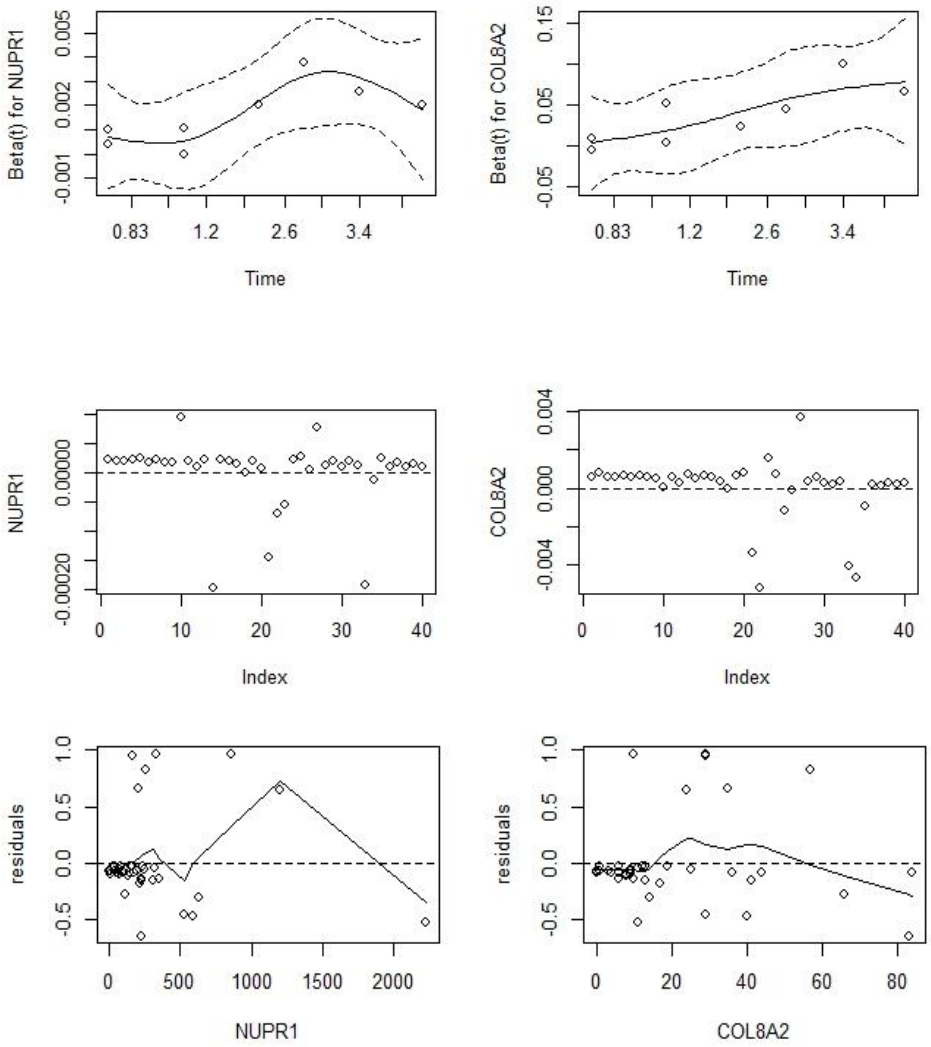


FIGURE A3: MODEL DIAGNOSTICS FOR RECURRENCE AFTER ADJUVANT RADIOTHERAPY MODEL INCLUDING NUPR1 AND COL8A2. A: SCHOENFELD RESIDUALS DO NOT SHOW ANY SYSTEMATIC TRENDS OVER TIME. **B:** INFLUENTIAL CASES PLOT, SHOWING THE ESTIMATED CHANGES IN THE REGRESSION COEFFICIENTS IF EACH CASE IN TURN WAS DELETED. **C:** MARTINGALE RESIDUALS TO ASSESS FOR NON-LINEARITY.