Circulating Biomarkers and Prognosis in Colorectal Cancer

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Abstract

Colorectal cancer is an important global health problem as the second most common cause of cancer deaths worldwide. The ability to risk-stratify patients according to predicted outcome is fundamental to patient management and historically the TNM staging system has formed the basis for this. As more is becoming understood about the role of the host’s immune response on cancer progression and its influence on outcome, there is a growing need to evaluate the potential role of circulating inflammatory cytokines as prognostic markers in colorectal cancer.

In chapter Two, the patterns of disease recurrence were evaluated in a cohort of 237 patients. Recurrences were observed most frequently within two years of surgery and at distant sites. The prognostic value of pre-treatment carcinoembryonic antigen (CEA), as the most widely used circulating prognostic biomarker, was also evaluated in a subset of this cohort. We found it to be predictive of overall and disease free survival, independent of TNM stage and through these findings, we established CEA as a benchmark against which novel approaches could then be compared.

Chapter Three comprises a systematic review whereby seven studies were found to evaluate the prognostic value of multiple cytokine analysis. A combined cytokine score was utilised in four studies although the individual cytokines used and the methods by which they were combined varied between studies. Some promise was shown by applying a multi-marker approach to circulating cytokines.

Chapters Four and Five were prospective studies. In Chapter Four, IL-6, IL-8, IL-1β and TNFα plasma levels were compared between subjects who were recruited into three
groups: healthy controls, stage II disease and stage IV disease. Plasma IL-6 and IL-8 were found to differ significantly between patients with stage IV disease and those without. Furthermore, when a combined IL8-CEA score was developed, the ability to distinguish between the three groups was enhanced, compared to any individual marker. In Chapter Five, we went on to evaluate the prognostic value of circulating cytokine markers in a longitudinal study. A panel of eight cytokines was selected, each with a foundation in experimental data linking their actions to colorectal cancer progression. These cytokines were measured in a cohort of 73 patients with non-metastatic colorectal cancer, including the combination of IL8 and CEA, developed in Chapter Four. Following a median duration of 18 months, combined scores composed of IL-8 and IL-10, when combined with CEA, were shown to improve marginally upon CEA alone in risk-stratifying the cohort by disease free survival. A number of limitations to the study were acknowledged including a relatively small sample size and short follow-up duration, preventing the feasibility of a multi-variate survival analysis.

Whilst we found a multi-marker approach, combining circulating inflammatory cytokines with CEA, to offer some promise in colorectal cancer prognostication, further study is necessary to evaluate the IL10-CEA score in a larger study sample over a five year follow-up duration.
Acknowledgements

I would like to express my sincere gratitude to my supervisors Associate Professors Peter Larsen and Elizabeth Dennett for their guidance and support as well as their hard work and patience in supervising me throughout this research. I appreciate the balance they struck in allowing me the independence to pursue my own lines of enquiry while providing me with assistance when I needed it.

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I am thankful for the encouragement of my parents who have always supported me in everything I do. I thank my wife Harini for a joyful life outside of research and for encouraging me to persevere through the challenges in my work.

Lastly, I would like to thank each of the patients who agreed to take part in these studies. I was humbled to see how many people wanted to help others by contributing to research even when confronted with a life-changing diagnosis themselves. This work is dedicated to them.
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<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>APR</td>
<td>Abdominal Perineal Resection</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone Marrow Derived Cells</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer Associated Fibroblast</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C motif Chemokine Ligand</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic Antigen</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG Methylation Pathway</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal Instability</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
</tr>
<tr>
<td>CRM</td>
<td>Circumferential Resection Margin</td>
</tr>
<tr>
<td>CRP</td>
<td>C Reactive Protein</td>
</tr>
<tr>
<td>CSS</td>
<td>Cancer Specific Survival</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating Tumour Cell</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif Chemokine Ligand</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DFS</td>
<td>Disease Free Survival</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>ESMO</td>
<td>European Society of Medical Oncology</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FOBT</td>
<td>Faecal Occult Blood Testing</td>
</tr>
<tr>
<td>G CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GM CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GRO</td>
<td>Growth Regulated Oncogene</td>
</tr>
<tr>
<td>HDN</td>
<td>High Density Neutrophil</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia Inducible Factor-1</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>I-CAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic Heart Disease</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Interferon gamma-induced Protein</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>ITC</td>
<td>Isolated Tumour Cells</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>LC-CRT</td>
<td>Long Course Chemo-Radiotherapy</td>
</tr>
<tr>
<td>LDN</td>
<td>Low Density Neutrophil</td>
</tr>
<tr>
<td>LMR</td>
<td>Lymphocyte Monocyte Ratio</td>
</tr>
<tr>
<td>M CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
</tbody>
</table>
MCP  Monocyte Chemotactic Protein
MDC  Macrophage-Derived Chemokine
MDSC  Myeloid Derived Suppressor Cell
mGPS  Modified Glasgow Prognostic Score
MHC  Major Histocompatibility Complex
MIP  Macrophage Inflammatory Protein
MM  Micro-metastases
MMP  Matrix Metalloproteinase
MMR  Mismatch Repair
MRI  Magnetic Resonance Imaging
MSI  Microsatellite Instability
NCCN  National Comprehensive Cancer Network
NF-KB  Nuclear Factor Kβ
NLR  Neutrophil Lymphocyte Ratio
NSAID  Non-Steroidal Anti-Inflammatory Drug
OS  Overall Survival
pCR  Pathological Complete Response
PDGF  Platelet Derived Growth Factor
PET  Positron Emission Tomography
PFS  Progression Free Survival
PIPER  Presentations, Investigations, Pathways, Evaluation and Rx
PLR  Platelet Lymphocyte Ratio
ROC  Receiver Operator Characteristic
Treg  Regulatory T cell
SCGF  Stem Cell Growth Factor
SC-RT  Short Course Radiotherapy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology and End Results</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour Associated Macrophage</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumour Associated Neutrophil</td>
</tr>
<tr>
<td>TCL</td>
<td>T Cell Lymphocyte</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumour Growth Factor</td>
</tr>
<tr>
<td>T(_H^1)</td>
<td>Type 1 Helper T cell</td>
</tr>
<tr>
<td>T(_H^2)</td>
<td>Type 2 Helper T cell</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour Microenvironment</td>
</tr>
<tr>
<td>TME</td>
<td>Total Mesorectal Excision</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour Node Metastasis</td>
</tr>
<tr>
<td>TTR</td>
<td>Time to recurrence</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>USPSTF</td>
<td>United States Preventative Services Task Force</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter One

Introduction
1.1 Colorectal Cancer

The colon and rectum in health

*Embryology and anatomy*

Together, the colon and rectum form the large intestine, beginning at the ileo-caecal junction and ending at the anal canal. Although variable, its length is approximately 1.5m in total and the rectum alone approximately 15cm. The recto-sigmoid junction signifies the transition between colon and rectum where three longitudinal muscular bands termed *taenia coli* that run along the length of the colon, fuse to invest the rectum as a single continuous layer\(^2\).

The right side of the colon – including the caecum, appendix, ascending colon and proximal two-thirds of the transverse colon - is derived from the embryonic midgut and its blood supply is via the superior mesenteric artery. The left side of the colon is comprised of the distal third of transverse, descending and sigmoid colon – in addition to the rectum and is derived from the embryonic hindgut and supplied by branches of the inferior mesenteric artery. Draining lymphatic vessels and nodes of the colon and rectum follow their arterial supply (Figure 1)\(^2,3\).

The ascending and descending colon are retroperitoneal structures, whilst the transverse and sigmoid colon are typically mobile on a mesentery. The upper rectum is covered by peritoneum anteriorly and laterally and anteriorly alone in the mid-rectum. Here, the anterior peritoneal reflection lies roughly 7.5cm from the anal verge in men forming the recto-vesical fold, and 5.5cm in women as the recto-uterine pouch, or Pouch of Douglas. The mesorectum is the perirectal areolar connective tissue which surrounds the rectum
beneath the peritoneal reflection down to the levator ani and is enveloped by the mesorectal fascia. The mesorectum is traversed by terminal branches of the inferior mesenteric artery and contains the draining perirectal lymphatics and lymph nodes of the rectum\(^4\).

Four tiers of draining lymph nodes of the colon and rectum have been described: epicolic, paracolic, intermediate and principal nodes, which are situated alongside the bowel, around the marginal blood vessels, along the main branches of the superior and inferior mesenteric artery and at the origin of the mesenteric arteries, respectively \(^5\).

Figure 1. Lymphatic drainage of colon and upper rectum. Image reproduced with permission from the publisher\(^5\).
Venous drainage of the colon and upper rectum is via the superior and inferior mesenteric veins which join to form the hepatic portal vein that subsequently enters the liver, branching to supply a central terminal venule to individual hepatic lobules. In contrast to this, venous drainage of the middle and lower rectum is into the systemic venous circulation, via the internal iliac and pudendal veins (Figure 2).

![Diagram of venous drainage](image)

Figure 2. Venous drainage from colon and rectum. Reproduced with the publishers permission.

**Function, Histology and Physiology**

The main functions of the colon are to reabsorb water and electrolytes within chyme from the small intestine. Absorption occurs across enterocytes, which form the most abundant cell type in colorectal mucosal epithelium. Also within the mucosal epithelium are mucin-containing goblet cells whose secretions line the colon with a moderately bicarbonate-rich
mucous; this allows easy passage of faecal material by lubrication and protects the mucosal lining from abrasion and acidic by-products of fermentation by commensal bacteria. Occasional enteroendocrine cells are found within the colonic and rectal epithelium and function mainly in feedback mechanisms with the proximal alimentary tract to either promote or inhibit motility and secretion.

Whilst absorption and fermentation does occur in the left side of the colon and the rectum, their function is more concerned with the storage of faeces until expulsion, the rectum being an expansive reservoir that is usually empty.

The gut flora of the large intestine is formed by several thousands of microbial species and various beneficial actions have been acknowledged in the scientific literature. These include, the provision of nutrients via metabolism of indigestible compounds, the prevention of colonisation by pathogens, and the production of vitamin K as a metabolic by-product.

**Epidemiology**

In 2012, the GLOBOCAN series of the International Agency for Research on Cancer published incidence and mortality data compiled on 184 countries worldwide from population-based cancer registries. In this report, colorectal cancer was found to be the third most common cancer behind lung and breast in both sexes combined, third behind lung and prostate in men and second behind only breast in women. It had the fourth highest mortality rate after lung, stomach and liver.

A key finding of the report was the high variability in incidence in Colorectal cancer (CRC), ten-fold, between regions of the world and New Zealand was identified as a country with
the highest incidence rate observed\textsuperscript{11}. In the year 2015, 3158 new diagnoses of CRC were made in New Zealand, with an age-standardised rate of 42.3 per 100 000 population \textsuperscript{12,13}.

The National Cancer Institute of the USA’s Surveillance, Epidemiology, and End Results (SEER) program provides incidence and survival trends which have demonstrated a 22% increase in incidence of colorectal cancer among adults aged below 50 years between 1992 and 2013 \textsuperscript{14}. A rising incidence of rectal cancer in younger patients has also been observed from Cancer Registry data within New Zealand although the cause of this trend is uncertain\textsuperscript{15}.

The sub-site distribution of cancers are approximately as follows, 40% in the proximal colon, 25-30\% in the distal colon and 30-35\% in the rectum\textsuperscript{14,16}. A relative increasing incidence of right-sided colon cancers and decreasing incidence of left-sided colon cancers, termed ‘left-to-right shift’ has been reported in analyses of temporal trends in National Cancer Registries both Internationally and within New Zealand\textsuperscript{16}.

**Risk Factors**

A number of hereditary and environmental factors have been linked to an increased likelihood of developing colorectal cancer. These are listed in Table 1.
Table 1. Risk factors for the development of colorectal cancer. Adapted from Haggar et al\textsuperscript{17}

<table>
<thead>
<tr>
<th>Modifiable</th>
<th>Non-Modifiable</th>
</tr>
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<tbody>
<tr>
<td>Diet</td>
<td>Age</td>
</tr>
<tr>
<td>Physical inactivity and Obesity</td>
<td>Personal history of adenomatous polyps</td>
</tr>
<tr>
<td>Smoking</td>
<td>Personal history of colorectal cancer</td>
</tr>
<tr>
<td>High alcohol intake</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Family History of colorectal cancer in first-degree relative or second-degree relatives diagnosed under 50yr</td>
</tr>
<tr>
<td></td>
<td>Familial adenomatous polyposis (FAP) (Figure 3)</td>
</tr>
<tr>
<td></td>
<td>Lynch Syndrome / Hereditary nonpolyposis colorectal cancer (HNPCC)</td>
</tr>
</tbody>
</table>

Figure 3. Opened colon specimen from a patient with familial adenomatous polyposis. Image reproduced with the publishers permission.\textsuperscript{18}
Carcinogenesis and Progression

Carcinogenesis

Carcinogenesis is a multistep process resulting in the accumulation of genetic mutations that may occur spontaneously, as a result of damage, or be inherited; the consequence being dysregulated clonal expansion\textsuperscript{19}. The mutations are primarily of proto-oncogenes and tumour suppressor genes, resulting in self-sufficient cellular growth and insensitivity to growth inhibition, respectively. Oncogene k-RAS activation is considered an early event in 40% of colorectal cancers \textsuperscript{20,21}. The tumour suppressor gene, p53, functions to prevent cell proliferation in the presence of DNA damage, stimulating DNA repair and apoptosis and is mutated in up to 70% of colorectal cancers \textsuperscript{22,23}. Another tumour suppressor gene, Adenomatous Polyposis Coli (APC), underlies 40-80% of sporadic CRCs in addition to causing familial adenomatous polyposis (FAP) if affected by germline mutation \textsuperscript{21,24,25}.

The baseline rate of genetic mutations is considered much too low to result in invasive adenocarcinoma and three distinct molecular pathways of genomic instability have been described that lead to colorectal cancer\textsuperscript{26}. The first and most common is chromosomal instability (CIN). Here, a chromosomal region or entire chromosome is gained or lost and this is seen in 70% of CRCs \textsuperscript{27}. The second pathway is microsatellite instability (MSI), which accounts for approximately 15% of sporadic CRC\textsuperscript{28,29}. It is also the hallmark of hereditary non polyposis coli (HNPCC)\textsuperscript{26,30}. Deficient mismatch repair proteins result in an increased frequency of mutations\textsuperscript{26}. MSI is associated with right-sided cancers, mucinous cell type and the presence of infiltrating lymphocytes\textsuperscript{26} and generally carries with it a more favourable prognosis\textsuperscript{31}. Thirdly, the CpG island methylation pathway (CIMP) results from widespread epigenetic hypermethylation of CpG islands\textsuperscript{30}. This may lead to genetic
silencing including of MMR gene promotors and result in MSI demonstrating that the three pathways may overlap in CRC\textsuperscript{30}.

\textit{Adenoma-Carcinoma Sequence}

Evidence of a sequence of progression from adenoma to carcinoma, a widely accepted model, comes from epidemiological, histopathological and genetic data\textsuperscript{32}. In terms of epidemiology, countries with high prevalence of colorectal cancer are also found to have high prevalence of adenomatous polyps, both increasing with advancing age and with a peak prevalence for adenomas that precedes cancer by roughly five years\textsuperscript{33, 34}. Histologically, adenomata have been found to contain foci of invasive cancer in 0.2-8.3\% of cases, providing evidence of malignant cells arising out of precursor adenoma lesions\textsuperscript{35, 36}.

![Figure 4. Adenoma-carcinoma progression with common genetic mutations. Reproduced with the publishers permission.](image)

An example of genetic evidence of the adenoma-carcinoma sequence are studies showing p53 mutations to occur within adenomas, invasive foci within adenomas and colorectal cancer, with increasing frequency, from 25\% to 50\% to 75\% respectively\textsuperscript{37} (Figure 4).
Serrated polyp-carcinoma sequence

A distinct pathway, termed the serrated polyp-carcinoma sequence is thought to underlie 7.5-10% of sporadic CRC. The cancers arise from serrated polyps which include hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas. Adenocarcinoma arising from serrated adenomas are more common in females and tend to be right-sided colon cancers.

Diagnosis and investigation

Clinical Presentation

A patient with colorectal cancer may come to medical attention through any of the following means: i) symptoms, ii) anaemia, iii) screening or surveillance investigation, or iv) incidental finding.

Symptoms

Presenting symptoms include change in bowel habit, abdominal pain, rectal bleeding and symptoms of anaemia i.e. fatigue, lethargy and shortness of breath. Blood loss per rectum is likely to be more overt the more distal the tumour producing blood that is brighter and less mixed with stool than bleeding from more proximal colonic cancers. Bleeding from right sided cancers may be entirely occult and detectable only through faecal occult blood testing or the detection of iron deficiency anaemia. Additionally, caecal and ascending colon cancers have been associated with greater daily blood loss than cancers elsewhere in the colon or rectum. Tenesmus, the sudden severe urge to defaecate in the absence of stool, and mucosy rectal discharge are symptoms seen more specifically with rectal cancer.

10
Patients presenting with advanced, metastatic disease may present with pain over the affected organ (e.g. right upper quadrant pain as a result of liver capsular pain, or vertebral bone pain), jaundice may be the presenting symptom of a patient with liver metastases resulting in biliary obstruction.

**Emergency Presentation**

Approximately 20% of patients with colorectal cancer present as an emergency, with intestinal obstruction, perforation or haemorrhage. Data from 82,000 patients collected through the National Bowel Cancer Audit in the United Kingdom, identified age below 50 or above 80 years, non-white ethnicity, high level of deprivation and particularly the presence of dementia as risk factors for emergency presentation.

**Screening & Investigation**

**Screening**

National screening programs are implemented with the goals of earlier detection of pre-existing colorectal cancer and reducing the incidence of new colorectal cancer through the removal of precursor polyps. Clear evidence demonstrates an improvement in survival in patients aged between 50 and 75 years through the use of screening with faecal occult blood testing (FOBT) and flexible sigmoidoscopy. The benefit is greatest with regular and frequent testing. Likewise, prevention of colorectal cancer through endoscopic polypectomy is also well-documented.

The United States Preventive Services Task Force recommends that people aged 50 years undergo screening for colorectal cancer. Data published in 2012 showed that 57.8% of patients underwent screening investigation. The following factors were associated
with being more likely to undertake screening: private health insurance, high income, college graduates, over 60 years old\textsuperscript{52}.

In New Zealand, the roll out of a national screening program began in July 2017 and is an invitation-based programme offering Faecal Occult Blood Testing (FOBT), with or without endoscopy, to those aged between 60 and 74 years old\textsuperscript{54}.

\textit{Investigation}

The investigation of choice for suspected colorectal cancer is colonoscopy\textsuperscript{55}. In addition to direct visualisation, this approach also allows polypectomy or biopsy, securing histological confirmation. A tattoo is often placed a few centimetres distal to the lesion to aid in surgical resection, particularly where the tumour is impalpable. These benefits are not conferred in cases where Computed Tomography (CT) colonography is undertaken although a meta-analysis of detection rates of colonoscopy and CT colonography demonstrated similar sensitivities between the two approaches: 96.1\% (95\% CI, 93.8-97.7) for CT colonography and 94.7\% (95\% CI, 90.4-97.2) for colonoscopy \textsuperscript{55}.

In cases where a complete pre-operative colonoscopy is precluded by an obstructing cancer, it is recommended that one is performed as soon after surgery as possible to detect potential synchronous cancers. A synchronous CRC is defined as a distinct lesion from the primary, diagnosed within six months of the initial cancer and is separated by normal bowel. A large population-based study in the Netherlands reported the incidence of synchronous CRC in 3.9\% of all newly diagnosed CRCs\textsuperscript{56}. Pre-operative identification of such lesions may influence the extent of colorectal resection.
The presence and extent of metastatic spread is evaluated by CT with intravenous and oral contrast, which also allows assessment of local invasion and involvement of adjacent structures. It should be noted that the sensitivity of CT to detect peritoneal nodules, nodal involvement and transmural mural invasion is limited.\(^{57}\)

Whilst CT of the chest, abdomen and pelvis is widely recommended to pre-operatively stage rectal cancer, CT of the chest is more controversial in colon cancer, where lung metastasis is relatively infrequent.\(^{58}\) Some guidelines recommend pre-operative chest X ray and CT chest only for suspicious lesions on X ray.\(^{59}\)

The main aims of loco-regional staging of rectal cancer is to assess the depth of tumour invasion, the presence and number of metastatic regional lymph nodes, the circumferential resection margin (CRM) and anal sphincter complex involvement. Two acceptable approaches include endoscopic ultrasound and T2 weighted magnetic resonance imaging (MRI).

Endoscopic ultrasound provides accurate assessment of the mucosa and submucosa, and has performed well in studies assessing T1 and T2 tumours with a sensitivity of 94% and specificity 98%.\(^{60,61}\) However, its accuracy diminished for more locally advanced tumours and unlike MRI, endoscopic ultrasound was unable to assess mesorectal fascia involvement, an important prognosticator. In contrast, MRI has been shown to have a sensitivity of 77% and specificity of 94% for predicting circumferential resection margin.\(^{62}\)

Suspicious lesions of the liver detected on CT may be further evaluated by liver MRI which has a proven benefit particularly for small lesions in the presence of hepatic steatosis, achieving an overall sensitivity of up to 97%.\(^{63}\)
There is conflicting evidence of a benefit from routine Positron emission tomography – computed tomography (PET/CT) in pre-operative staging. Whilst FDG PET/CT has been shown to have a similar sensitivity to CT in detecting liver metastases of 78-95%, studies have demonstrated a superior sensitivity in the detection of extra-hepatic disease. Due to its poor specificity however, the role of FDG PET/CT is generally limited to the assessment of extra-hepatic disease in patients being worked-up for radical treatment of colorectal liver metastases. However, the role for PET/CT in suspected recurrence is more convincing, particularly in preventing unnecessary laparotomy.

**TNM Staging System**

In 1932, Cuthbert Dukes, a London pathologist, published his classification of rectal cancer which became one of the earliest colorectal cancer staging systems to be adopted. In it, Dukes’ stage A indicates invasion into the bowel wall and Dukes B invasion through it. Dukes C indicates lymph node involvement. The Dukes classification was later modified by Gabriel et al in 1935 to subdivide Dukes C into C1 and C2 depending on whether regional lymph nodes only or apical lymph nodes were involved, respectively. It was not until 1968 when the Dukes classification was further modified to include Stage D, representing distant metastasis.

Michigan researchers Astler and Coller published their own modifications to the original Dukes classification in 1954, which became known as the Astler-Coller classification. Stage A were lesions limited to the mucous membrane, B1 were those limited to muscularis propria, B2 those extending beyond muscularis propria. Stages C1 and C2 corresponded to B1 and B2 but with lymph node involvement and stage D represented distant metastasis.
In current practice, pre-operative staging follows ‘TNM classification’ as laid out by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) headquartered in Geneva, Switzerland. ‘T’ refers to the primary tumour and advancing T stage relates to increasing depth of invasion so that T3 has breached muscularis propria and T4 breaches serosa. The ‘N stage’ refers to regional lymph node status with advancing N stage relating to an increasing number of lymph nodes found to contain malignant cells. The M stage refers to the presence of distant metastatic spread (Table 2).

The 7th edition of the TNM staging classification was published in the year 2009 and applies to diagnoses 2010-2016. The 8th edition staging manual was published in year 2016 for application to cancer diagnoses in 2017 onwards.¹
Table 2. TNM staging criteria for colorectal cancer taken from AJCC staging manual 8th edition.  

<table>
<thead>
<tr>
<th>T Stage (Primary tumour)</th>
<th>N Stage (Regional Lymph nodes)</th>
<th>M Stage (Distant Metastasis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx - Primary tumour cannot be assessed</td>
<td>Nx - Regional lymph nodes cannot be assessed</td>
<td>M0 - No distant metastasis</td>
</tr>
<tr>
<td>T0 - No evidence of primary tumour</td>
<td>N0 - Regional lymph node metastasis</td>
<td>M1 - Distant metastasis present</td>
</tr>
<tr>
<td>Tis - Carcinoma in situ: intraepithelial or invasion of lamina propria</td>
<td>N1 - 1-3 regional nodes affected</td>
<td>M1a - Confined to one organ or site (e.g. distant lymph nodes, liver, lung)</td>
</tr>
<tr>
<td>T1 - Invades submucosa</td>
<td>N1a - One regional node</td>
<td>M1b - More than one organ/site</td>
</tr>
<tr>
<td>T2 - Invades lamina propria</td>
<td>N1b - 2-3 regional nodes</td>
<td>M1c - Peritoneal metastasis</td>
</tr>
<tr>
<td>T3 - Invades muscularis propria into pericolic tissues</td>
<td>N1c - Tumour deposit(s) in subserosa, mesentery, or nonperitonealised pericolic or perirectal tissues without regional nodal metastasis</td>
<td></td>
</tr>
<tr>
<td>T4a - Penetrates to surface of the visceral peritoneum</td>
<td>N2 - 4 or more nodes</td>
<td></td>
</tr>
<tr>
<td>T4b - Directly invades or is adherent to other organs or structures</td>
<td>N2a - 4-6 regional nodes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2b - 7 or more regional nodes</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. AJCC Stage group according to AJCC staging manual, 8th edition. 

<table>
<thead>
<tr>
<th>T Stage</th>
<th>N Stage</th>
<th>M Stage</th>
<th>Stage Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
<td>0</td>
</tr>
<tr>
<td>T1-2</td>
<td>N0</td>
<td>M0</td>
<td>I</td>
</tr>
<tr>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>IIA</td>
</tr>
<tr>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
<td>IIB</td>
</tr>
<tr>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
<td>IIC</td>
</tr>
<tr>
<td>T1-2</td>
<td>N1/N1c</td>
<td>M0</td>
<td>IIIA</td>
</tr>
<tr>
<td>T1</td>
<td>N2a</td>
<td>M0</td>
<td>IIIA</td>
</tr>
<tr>
<td>T3-T4a</td>
<td>N1/N1c</td>
<td>M0</td>
<td>IIIB</td>
</tr>
<tr>
<td>T2-3</td>
<td>N2a</td>
<td>M0</td>
<td>IIIB</td>
</tr>
<tr>
<td>T1-2</td>
<td>N2b</td>
<td>M0</td>
<td>IIIB</td>
</tr>
<tr>
<td>T4a</td>
<td>N2a</td>
<td>M0</td>
<td>IIIC</td>
</tr>
<tr>
<td>T3-4a</td>
<td>N2b</td>
<td>M0</td>
<td>IIIC</td>
</tr>
<tr>
<td>T4b</td>
<td>N1-2</td>
<td>M0</td>
<td>IIIC</td>
</tr>
<tr>
<td>Any T</td>
<td>Any N</td>
<td>M1a</td>
<td>IVA</td>
</tr>
<tr>
<td>Any T</td>
<td>Any N</td>
<td>M1b</td>
<td>IVB</td>
</tr>
<tr>
<td>Any T</td>
<td>Any N</td>
<td>M1c</td>
<td>IVC</td>
</tr>
</tbody>
</table>

The eighth edition remains similar to the seventh with two main changes. Firstly, stage M1c was included which denotes the presence of peritoneal metastasis. Its inclusion as a separate stage to M1b came as a result of studies showing peritoneal involvement to carry significantly worse prognosis77,78 (Table 3). The second change relates to the presence of micrometastases (MM) within regional lymph nodes. Defined as clusters of 10 to 20 tumours cells or clumps of tumour on cut section that measure ≥0.2mm in diameter, MMs found within lymph nodes are considered positive according to the eight edition 1.

Previous editions drew no distinction between the presence of isolated tumour cells (ITCs) and MM leading to conflicting results and confusion79,80. More recently, a meta-analysis
and pooled analysis of five studies has found the presence of lymph node micrometastases to predict a significantly higher likelihood of disease recurrence in stage I and II colorectal cancer (OR 4.94, 95% CI 1.69-14.46) compared to those with isolated tumour cells\textsuperscript{81}. Patients with ITCs did not have an increased risk of recurrence to those without (OR 1.00, 95% CI 0.53-1.88)\textsuperscript{81}.

**Surgery**

**Operations**

Surgical resection of the primary tumour with clear margins and regional lymph nodes forms the cornerstone for cure for loco-regional disease (Figure 5). Which operation is performed relies largely on the location of the tumour and Table 4 summarises potentially curative options available by tumour site.
Table 4. Choice of curative operation, based on primary tumour location.

<table>
<thead>
<tr>
<th>Location of tumour</th>
<th>Operation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix</td>
<td>Right hemicolecotomy</td>
</tr>
<tr>
<td>Caecum</td>
<td>Right hemicolecotomy</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>Right hemicolecotomy</td>
</tr>
<tr>
<td>Hepatic flexure</td>
<td>Right hemicolecotomy</td>
</tr>
<tr>
<td>Proximal transverse colon</td>
<td>Right or Extended right hemicolecotomy</td>
</tr>
<tr>
<td>Distal transverse colon</td>
<td>Extended right hemicolecotomy</td>
</tr>
<tr>
<td>Splenic flexure</td>
<td>Left hemicolecotomy or extended right</td>
</tr>
<tr>
<td>Descending colon</td>
<td>Left hemicolecotomy</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>Sigmoid colectomy / high anterior resection*</td>
</tr>
<tr>
<td>Recto-sigmoid junction</td>
<td>High anterior resection*</td>
</tr>
<tr>
<td>Upper rectum</td>
<td>Low anterior resection*</td>
</tr>
<tr>
<td>Mid-rectum</td>
<td>Low anterior resection*</td>
</tr>
<tr>
<td>Lower rectum &gt;2cm from anal</td>
<td>Ultra low anterior resection*</td>
</tr>
<tr>
<td>Lower rectum &lt;2cm from anal</td>
<td>Abdominal Perineal Resection</td>
</tr>
<tr>
<td>Multiple sites</td>
<td>Subtotal colectomy or proctocolectomy</td>
</tr>
</tbody>
</table>

*or Hartmann's procedure (end colostomy)
Figure 5. Intra-operative photograph, right hemicolecotomy. Following the resection of the right colon, the divided ends of the terminal ileum (white arrow) and transverse colon (yellow arrow) are joined together using a linear stapling device. Image reproduced with the publishers permission. 82

_Total Mesorectal Excision_

The surgical approach of resecting rectal cancers en bloc with the surrounding mesorectum was first described by Bill Heald in 1979 and has since revolutionised rectal cancer surgery83 (Figure 6). It is based on the principle that the mesorectum contains the draining lymphovasculature to the tumour and by employing this approach, Heald achieved local recurrence rates as low as 3.7%84.
Post-operative systemic therapies

For colon cancer, the benefits of adjuvant chemotherapy have been widely accepted in patients with stage III disease where there is a 30% relative risk reduction. A 5-FU Oxaliplatin-based regime (FOLFOX) is generally recommended and Capecitabine is considered an oral alternative with similar survival benefits. Amongst the evidence supporting routine adjuvant chemotherapy following surgery for stage III disease is a systematic review and two RCTs demonstrating an improved disease free survival and overall survival.

By contrast, the evidence in support of routine treatment in stage II colon cancer is much scarcer. Four RCTs comparing adjuvant therapy with surgery alone for stage II colon cancer have shown no clear survival benefit and cancer societies including the American Society of Clinical Oncologists (ASCO), the European Society of Medical Oncology (ESMO),
and National Comprehensive Cancer Network (NCCN) do not recommend routine adjuvant chemotherapy in these patients. Whilst these bodies have named a number of ‘high-risk’ features that may influence the decision of offer treatment the evidence for survival benefit in this sub-group is also weak 93:

- Lymph node yield below 12
- pT4 tumours
- Obstruction or perforation
- Lymphovascular and perineural invasion
- Histologically high grade tumour

In rectal cancer, the NCCN recommends routine adjuvant chemotherapy in all patients that underwent neoadjuvant chemoradiation 94. This is based on expert consensus although a meta-analysis of four European trials was inconclusive in proving a significant benefit 95.

**Surveillance and follow up**

The main aim of post-operative surveillance is to detect otherwise clinically silent recurrences. In the case of liver or lung oligometastatic disease, this may mean potentially curative surgery can be offered 96, with five-year survival rates approaching 40% 97, 98. A Cochrane review published in 2015 included 53 studies evaluating the ability of carcinoembryonic antigen (CEA) to detect post-operative recurrences in colorectal recurrence and came to the conclusion that CEA is insufficiently sensitive to be used alone 99. Most cancer societies recommend both CEA and imaging modalities in post-operative surveillance 100-102.
The New Zealand Guidelines Group made six recommendations for post-operative surveillance in their document ‘management of early colorectal cancer’ 12 published in 2011, which are:

- Colon and rectal cancer patients that did not undergo complete colonoscopy before surgery should be offered colonoscopy within six months of discharge.
- Clinical assessment should be undertaken yearly for suggestive symptoms of relapse.
- High-risk patients should have a clinical review every six months to one year for the first three years, then yearly for at least five years.
- Low-risk patients should have a colonoscopy every three to five years.
- Clinical assessment for colon cancer patients should include CEA, chest, abdominal and pelvic CT scans, or liver ultrasound and colonoscopy.
- Clinical assessments for rectal cancer patients should include CEA, abdominal and pelvis CT scans, colonoscopy and proctoscopy or sigmoidoscopy.

**Prognostic Predictors**

Whilst the TNM staging system is recognised as the strongest and most widely established prognostic predictor of colorectal cancer, a number of additional clinical-pathological factors have been shown to yield prognostic value.

Emergency presentation.

Patients presenting with perforation, bleeding or obstruction have been found to exhibit poorer five-year survival compared with their counterparts undergoing planned care. In their Scottish study, McArdle et al compared the outcomes of 986 patients presenting as
an emergency to 2214 of those presenting non-emergently and found the adjusted hazard ratio for overall survival to be 1.68 (95% CI, 1.49-1.90, P<0.001)\textsuperscript{103}.

Histological grade.

High histological grade refers to poorly differentiated tumours and is associated with worse outcomes. A disadvantage to clinical application of this factor is its subjectivity and inter-observer variability\textsuperscript{104}.

Lymphovascular and Perineural Invasion.

The prognostic value of lymphovascular\textsuperscript{105} and perineural invasion\textsuperscript{106} is well-established in colorectal cancer and forms an integral part in the assessment of the malignant polyp especially, where the presence of these prognostic markers influence which patients may be offered surgery.

Mucinous adenocarcinoma.

Mucinous adenocarcinoma is when greater than 50% of a lesion of composed of extracellular mucin\textsuperscript{107}. These tumours are associated with right-sided colonic tumours and advanced stage at presentation\textsuperscript{108}. A 2012 meta-analysis included 35 studies in a survival analysis and found mucinous histology to be an risk factor for recurrence and death\textsuperscript{109, 110}.

Mismatch Repair (MMR) protein deficiency.

Deficiency of one or more mismatch repair proteins (MLH1, MSH2, MSH6) is seen in patients with Lynch syndrome and 15% of those with sporadic colorectal cancer. This results in a high amount of microsatellite instability and is associated with improved five-year overall and cancer-specific survival\textsuperscript{111}.
Lymph Node Yield.

A lymph node yield of at least twelve is thought to represent an optimal mesenteric resection and accurate lymph node stage and is therefore recommended in the AJCC staging manual, 8th edition. Diligence and experience of pathology personnel is often an acknowledged confounder in this parameter.\(^1,^{112}\)

Lymph Node Ratio.

The number of positive lymph nodes as a ratio of total lymph node yield has been demonstrated to be a powerful prognostic value. Ceelen et al performed a systematic review that included 16 studies of patients with stage III colon and rectal cancers. They found lymph node ratio not only to be an independent predictor of overall and disease-free survival but to be superior to lymph node yield.\(^{113}\)

Isolated Tumour Deposits.

Tumour deposits are nodules within pericolonic or perirectal tissue away from the leading edge of the tumours and with no evidence of residual lymph node. They have been found to occur in 2.5% of colon cancers and 3.3% of rectal cancers in the absence of positive lymph nodes.\(^{114}\) These were previously considered as part of the ‘T’ stage but since the AJCC seventh edition now form part of the ‘N’ stage. While studies have found this factor to be associated with poorer overall and disease-free survival there are a number of interpretation challenges. These include differentiation from discontinuous tumour spread, totally replaced lymph node and venous invasion.\(^{112}\)

Circumferential Resection Margin and Tumour Regression Score in Rectal Cancer.
Prognostic factors that are unique to rectal cancer include the circumferential resection margin, and tumour regression score following neo-adjuvant treatment. A distance of 1mm or less between the tumour and circumferential resection margin has been shown consistently to predict a higher rate of local and distant recurrence\textsuperscript{1,115}. The status of the circumferential resection margin largely, but not exclusively, determines the extent of residual tumour in local-regional disease. As defined in the AJCC staging manual R0 denotes no residual tumour, R1, microscopic residual tumour and R2 macroscopic residual tumour\textsuperscript{116}.

BRAF.

The BRAF V600E mutation is present in nearly 10% of CRCs. An association has been observed between BRAF mutation and female sex, high grade, advanced age and MSI-H tumours. The BRAF V600E mutation confers a survival disadvantage among patients with microsatellite stable (MSS) disease. Contrastingly, a survival advantage has been observed among patients with MSI-H disease\textsuperscript{117}.

KRAS.

Mutations in the Kirsten RAS proto-oncogene (KRAS) have been observed in 30-40% of CRCs. The presence of KRAS mutation has been shown to predict response to anti-GFR therapy in metastatic disease\textsuperscript{118}.

12-gene Recurrence Score assay.

The Oncotype DX Colon Recurrence Score assay utilises reverse transcription –polymerase chain reaction on primary colon tumour tissue. The score was initially developed and validated in stage II colon cancer but has since been validated in stage II/III disease treated
with adjuvant chemotherapy\textsuperscript{119}. The SUNRISE study in Japan found the Oncotype DX Colon Recurrence Score assay to independently predict recurrence in stage II/III colon cancer patients who were treated with surgery alone\textsuperscript{120}.

Serum Carcinoembryonic Antigen.

The prognostic value of preoperative CEA has been observed in clinical studies as early as the 1970’s\textsuperscript{121}. In 1984, Wolmark et al published the pooled results from two National Surgical Adjuvant Breast and Bowel Project (NSABP) trials, based in the USA. They reported the preoperative CEA to be associated with disease recurrence in patients with Dukes B and C disease\textsuperscript{122}. A number of other early studies have also demonstrated an observed association between raised preoperative CEA and recurrence in colorectal disease although many were limited by small study samples\textsuperscript{121,123,124}.

In the College of American Pathologists Consensus Statement 1999, preoperative CEA was stratified as a ‘Category I’ prognostic factor in colorectal carcinoma by a multidisciplinary panel including Oncologists, Pathologists and Statisticians\textsuperscript{125}. This indicates the strength of evidence in the available literature at the time. Whilst the incorporation of preoperative CEA into the TNM staging system was suggested as the ‘C’ stage, it was not adopted in subsequent AJCC staging manuals. A major reason for this was disagreement over the optimum cut off value\textsuperscript{126}. Contemporary studies have since set out to re-evaluate the prognostic value of preoperative CEA in the context of modern chemotherapy, although a single cut-off value remains to be universally validated and agreed upon. This point is exemplified by the USA-based study of Thirunavukarasu et al who analysed data from the National Cancer Institute’s SEER database of 16,619 patients with colonic cancer\textsuperscript{127}. They found the ‘C stage’ to significantly effect 5-year survival when
incorporated into the TNM staging system. However, a raised CEA was not determined by a pre-defined value but by what the reporting physician identified as elevated\textsuperscript{128}.

Konishi et al’s 2018 retrospective study examined the prognostic value of pre- as well as post-operative CEA in 1027 patients with stage I-III colon cancer undergoing curative resection\textsuperscript{129}. Patients with a raised pre-operative CEA that normalised after surgery were found to have a similar prognosis to patients with a normal pre-operative CEA. Although these results cast doubt on the future utility of pre-operative CEA, a number of questions about the prognostic role of post-operative CEA remain unanswered. These include, the optimal timing of post-operative measurement in relation to adjuvant therapy and the confounding effect of a complicated post-operative course. Further studies are required to compare the prognostic value of preoperative CEA in patients with raised and normal post-operative CEA in order to address these questions.

In routine clinical practice around the world, a pre-operative CEA is often not measured. Therefore, retrospective analysis is often hampered by a large degree of missing data. This is demonstrated in the study of Becerra and colleagues who included 137,381 patients with stage I-III colon cancer from the National Cancer Database\textsuperscript{130} where pre-operative CEA values were available in only 50.6% of the cohort.

Studies such as that of Sasaki et al demonstrate the prognostic value of pre-operative CEA in stage IV colorectal cancer patients undergoing surgery with curative intent. Their study of 484 patients undergoing surgery for colorectal liver metastases were included and found an optimum cut off of 70 ng/mL for recurrence and 50ng/mL for survival\textsuperscript{131}. Similarly, pre-operative CEA has been shown consistently to predict prognosis in patients undergoing pulmonary metastectomy\textsuperscript{132}. 


Risk Assessment Models

There is a shift toward delivering personalised treatment whereby the available data be it clinical, radiological, histopathological, molecular or genetic is evaluated to accurately risk stratify patients and tailor treatment accordingly. A number of multi-variable risk assessment models have been developed to predict prognosis in colorectal cancer and twenty-nine such models were identified in a systematic review by the AJCC Precision Medicine Core (PMC)\(^1\). Following the application of stringent inclusion and exclusion criteria, three of these risk assessment tools were endorsed by the AJCC in their 8\(^{th}\) edition staging manual. One tool is for use in stage III colon cancer\(^{133}\), one for predicting survival following curative colectomy\(^{134}\) and a third for predicting outcome in locally advanced rectal cancer\(^{135}\).

Renfro et al’s 2014 prognostic tool was developed using data from 15 936 stage III patients accrued through 12 randomised clinical trials contained in the Adjuvant Colon Cancer End Points (ACCENT) database. The tool uses the following variables: age, sex, race, BMI, performance status, T stage, lymph node ratio, grade, chemotherapy agent and tumour location. External validation studies have shown the model to perform well in predicting overall survival although the predicted 3-year time to recurrence (TTR) of 80.3% in their external validation study fell outside the 95% confidence interval of the observed TTR, which was 74.8-78.7%\(^{133}\).

Weiser et al’s 2011 model is based on data from 128, 853 primary colon cancer patients from the SEER database. The factors included in the model are age, sex, T stage, grade, total lymph nodes and number of positive lymph nodes. In their study, Weiser et al found
their risk assessment model to predict five-year survival with 72% sensitivity and 64% specificity\textsuperscript{134}.

Valentini et al’s 2011 pooled data from five European clinical trials to include 2795 patients with locally advanced rectal cancer. Multivariate nomograms were developed to predict 5-year local recurrence, distant metastases and overall survival. The variables included in the risk assessment model for distant metastases, for example, are N stage, T stage, surgical procedure and administration of adjuvant chemotherapy\textsuperscript{135}. 
1.2 Inflammation and Colorectal Cancer

Population-based evidence for a link between inflammation and colorectal cancer

It has been estimated that 15-20% of the global cancer burden is attributable to, or associated with, underlying infection and/or inflammation\(^\text{136,137}\). Examples of this are widespread and include: hepatitis and hepatocellular carcinoma\(^\text{138}\); human papilloma virus and anal and cervical carcinoma\(^\text{139,140}\); chronic pancreatitis and pancreatic adenocarcinoma\(^\text{141}\); chronic prostatitis and prostate carcinoma\(^\text{142}\); endometriosis and endometrial adenocarcinoma\(^\text{143}\); thyroiditis and papillary thyroid cancer\(^\text{144,145}\). Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer\(^\text{146,147}\) and the observations that cancer risk is associated with the duration\(^\text{148}\) and severity\(^\text{149}\) of disease suggest a causal relationship. Some have suggested a shared genetic predisposition to underlie both conditions although a Swedish study of over 30,000 patients with inflammatory bowel disease found that the risk of colorectal cancer in their first-degree relatives was not significantly increased\(^\text{150}\).

Further evidence of a link between inflammation and colorectal cancer comes from population studies that have shown a reduced incidence of colorectal cancer\(^\text{151}\) amongst patients taking regular non-steroidal anti-inflammatory drugs (NSAIDs)\(^\text{152}\). It should be noted that some of these have been based upon secondary analyses and include large studies that were designed to evaluate the risks and benefits of NSAIDs in reducing cardiovascular risk. Prospective studies have demonstrated a reduction in the risk of colorectal cancer in patients taking aspirin or non-aspirin NSAIDs compared to those receiving a placebo, although the effect is often not seen until at least ten years of
therapy\textsuperscript{153-166}. The risks associated with long-term NSAID use are currently considered to outweigh the benefits of reducing the incidence of colorectal cancer in the general population. However, the US preventative services task force (USPSTF) recommend low-dose aspirin for primary prevention of cardiovascular disease and colorectal cancer in patients aged 50-59 years old with a greater than 10\% 10-year cardiovascular disease risk\textsuperscript{157}.

**Inflammation within the tumour microenvironment**

**Immune and stromal cell populations**

In 1863, Rudolf Virchow described an immune cell “lymphoreticular” infiltrate within tumours and first proposed this host immune response to play an important role in the origin of cancer\textsuperscript{158}. Subsequently, numerous populations of immune and inflammatory cells have been observed and identified within the tumour microenvironment (TME) of sporadic, as well as colitis-associated, CRCs, including: tumour associated macrophages (TAMs)\textsuperscript{159}, dendritic cells\textsuperscript{160}, tumour-associated neutrophils (TANs)\textsuperscript{161} and T cell lymphocytes\textsuperscript{162}. Whilst the TME is generally considered supportive of tumour growth and progression, some cellular subtypes display predominantly anti-tumour immune activity, namely adaptive immune cells including cytotoxic T lymphocytes\textsuperscript{163} and their abundance within the microenvironment is associated with favourable prognosis\textsuperscript{162}. This illustrates the complex interplay between tumour and host and its influence on tumour progression.

**Tumour-associated macrophages (TAMs)**

Macrophages are one of the most abundant inflammatory cell-type within the TME, making up to 80\% of the tumour mass\textsuperscript{158,164}. Tumour and stromal cells secrete chemo-
attractants such as C-C motif ligand 2 (CCL2), recruiting circulating monocytes into the
tumour. Here, monocytes differentiate into macrophages under the influence of
macrophage colony stimulating factor (M-CSF), also produced by tumour cells. A body
of experimental evidence now exists in support of there being two main subtypes of
macrophage within the TME, M₁ and M₂ with generally opposing functions. On the one
hand, M₁ macrophages are ‘classically activated’ and have anti-tumour functions, whilst
M₂ TAMs are ‘alternatively activated’ and have functions that are supportive to tumour
growth and progression. The predominant phenotype is largely influenced by the
profile of cytokines and chemokines within the tumour microenvironment and TAMs
demonstrate plasticity so are not committed to one phenotype but may alter with the
changing biochemical landscape. For example, granulocyte monocyte colony
stimulating factor (GM-CSF) and interferon γ (IFNγ) have been shown to stimulate
development of cytotoxic M₁ macrophages. The location of TAMs is also important as
studies have shown intra-tumoural TAMs to increase in density with advancing stage and
worsening prognosis whereas increasing numbers of peri-tumoural TAMs correspond
with tumours bearing favourable outcome. Some have interpreted this as peri-tumoural
TAMs to be reflective of an initial immune response that is protective to the host and as
macrophages infiltrate deeper into the tumour they are ‘entrained’ to take on pro-tumour
characteristics.

Tumour-associated neutrophils (TANs)

Chemo-attractants released by tumour cells, including interleukin 8 (IL-8), G-CSF and GM-
CSF, attract circulating neutrophils into the tumour where there is evidence that, like
macrophages, there exist two sub-types within the TME. N₁ neutrophils are primarily
cytotoxic toward tumour cells\textsuperscript{174} and stimulate a local anti-tumour immune response, mediated by T helper 1 (T\textsubscript{H}1) cells, through the release of TNF\textalpha, IL-12, GM-CSF and vascular endothelial growth factor (VEGF)\textsuperscript{174}. The N\textsubscript{2} phenotype is activated by tumour- and macrophage-derived factors particularly Tumour Growth Factor-\beta (TGF\beta), VEGF and IL-10 resulting in a supressed adaptive immune response, thus acting in favour of tumour growth and progression\textsuperscript{175}. N\textsubscript{2} neutrophils also act directly to promote tumour growth by releasing pro-angiogenic factor VEGF-A and tumour growth factors such as TGF\beta, epidermal growth factor (EGF) and platelet derived growth factor (PDGF)\textsuperscript{173,175}. N\textsubscript{2} neutrophils are also thought to possess pro-metastatic properties and an example of this is through the release of matrix metalloproteinase 9 (MMP-9), which in turn leads to extracellular matrix degradation: a necessary step in tumour invasion and intravasation\textsuperscript{176,177}.

\textit{Dendritic Cells (DCs)}

Dendritic cells (DCs) play an important role in adaptive immunity as antigen presenting cells (APCs)\textsuperscript{178}. In colorectal cancer, DCs are thought to either migrate to lymph nodes where they mature, or mature at the tumour edge itself, where they present cancer-associated antigens in order to activate an adaptive, T cell-driven, immune response\textsuperscript{179,180}. A potential pathway for tumours to evade immune-detection is by inhibiting the maturation of DCs. Michielson et al showed that tumour conditioned media from colorectal tumour explants inhibited dendritic cell maturation in response to lipopolysaccharide exposure and suggested that this was mainly mediated by CXCL and VEGF from the TME\textsuperscript{181}. 

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**Cancer Associated Fibroblasts (CAFs)**

Fibroblasts form the main cellular component of the tumour stroma and are recruited into the TME where they may differentiate into cancer-associated fibroblasts (CAFs) under the influence of cytokines including TGFβ, PDGF, and IL-6\(^1\). Unlike for TAMs, TANs and DCs, where evidence exists for both tumour-promoting and tumour-suppressing actions, studies on CAFs have demonstrated predominantly tumour-promoting effects\(^1\). These can be through stimulating tumour cell proliferation and survival through release of growth factors including hepatocyte growth factor (HGF), PDGF, fibroblast growth factor 1 (FGF-1)\(^1\); angiogenesis through VEGF release, and invasion and metastasis through TGFβ and MMP-9 release\(^1\).

**Cytotoxic T Cell Lymphocytes (CTLs)**

An increased density of T cell lymphocytes within the TME indicates the presence of a strong adaptive immune response and has been shown to be an indicator of favourable outcome\(^1\). The ‘immunoscore’, developed by Galon et al, is a validated prognostic score based on the density of CD3+ T cells, CD8+ cytotoxic T cells and CD45RO+ memory T cells within the central tumour and/or invasive margin\(^1\) and some studies have shown immunoscore to outperform traditional TNM staging in predicting outcome in colorectal cancer\(^1\). This shows the important influence that host immune response has on prognosis.

**T Helper Cells**

Whilst the role of CD8+ cytotoxic T cells in cancer is generally restricted to tumour cell clearance, CD4+ T helper cells have much more diverse functions\(^1\). Evidence supports
the presence of two main sub-types of T helper cells each with opposing actions within the TME \(^{188}\). Polarisation toward \(T_H1\) or \(T_H2\) differentiation depends largely on the profile of cytokine molecules within the microenvironment \(^{188}\). Whilst other T helper sub-sets found within the TME such as \(T_H17\) and \(T_{reg}\) cells exhibit plasticity and have mainly regulatory actions, \(T_H1\) and \(T_H2\) cells commit to one lineage or the other once differentiation has occurred and together, they represent a polarity that may profoundly impact tumour growth and progression \(^{188, 189}\).

Polarisation of T helper cells may be toward a predominantly \(T_H1\)- or \(T_H2\)-response. Each type of response is associated with a distinct profile of cytokines and cellular subtypes that effect tumour growth and progression in different ways (Table 5).

Table 5. The immune response to cancer within the tumour microenvironment.

<table>
<thead>
<tr>
<th>T Helper Cell Response</th>
<th>Cytokines</th>
<th>Cellular sub-types and Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T_H1) Response (Classical)</td>
<td>GM-CSF, TNFα, IFNγ, IL-2, IL-12</td>
<td>Stimulates an anti-tumour immune response through activation adaptive immune cells. These include, cytotoxic CD8+ T cells, M(_1) cytotoxic macrophages, N(_1) neutrophils and mature antigen presenting dendritic cells.</td>
</tr>
<tr>
<td>(T_H2) Response (Alternative)</td>
<td>IL-4, IL-5, IL-10, IL-13, TGFβ</td>
<td>Supresses the adaptive immune response. Stimulates M(_2) and N(_2) cells to promote tumour growth, angiogenesis, ECM degradation and invasion.</td>
</tr>
</tbody>
</table>

\(T_H1\) cell differentiation is stimulated by IFNγ and IL-12 and, through the release of cytokines including IFNγ, IL-2 and IL-12, exert a direct cytotoxic effect on tumour cells.
They also help to rally a CD8+ cytotoxic response with the cumulative effect of hindering cancer development\textsuperscript{190}. In contrast to this, T\textsubscript{H}2 cells release IL-4, IL-5, IL-10 and IL-13 which inhibit T cell-mediated cytotoxicity\textsuperscript{191} and are considered to contribute to a more tumour-permissive, immunosuppressed TME\textsuperscript{188}.

In addition to their role in tumour cell clearance, there is evidence that T\textsubscript{H}1 cells also influence cells of the innate immune system to differentiate into anti-tumour phenotypes such as M\textsubscript{1} macrophages, N\textsubscript{1} neutrophils and mature antigen presenting dendritic cells, or their corresponding pro-tumour phenotypes in the case of T\textsubscript{H}2 cells\textsuperscript{164, 192}. Macrophages may differentiate into M\textsubscript{1} or M\textsubscript{2} and neutrophils into N\textsubscript{1} or N\textsubscript{2} phenotypic subtypes. Unlike macrophages and neutrophils, which exhibit some plasticity between subtypes, T\textsubscript{H}1 and T\textsubscript{H}2 subtypes of T helper cells are committed to their lineage of differentiation (Figure 7).
Cytokines within the microenvironment

A vast number of cytokines are found within the tumour microenvironment and are products of tumour and stromal cells as well as those of the innate and adaptive immune systems discussed above. Whilst many of the central pro-inflammatory cytokines help to foster a primarily tumour-permissive microenvironment, others function to amplify and mediate anti-tumour effects. Others still have dual functions and do not sit neatly into a pro- or an anti-tumour group. Experimental evidence on some of the specific functions and downstream activation pathways are set out below for a selection of the most widely studied cytokines in CRC.
Interleukin 1β (IL-1β)

Interleukin 1β is a potent activator of the pro-inflammatory transcription factor, nuclear factor Kβ (NF-KB) and is considered to be one of the main inflammatory cytokines. NF-kB activation can occur in tumour cells, epithelial cells or inflammatory cells and its downstream actions include the further expression of cytokines, adhesion molecules and angiogenic factors with pro-tumour effects. Saccani et al demonstrated that while it is usually under tight control by its inhibitor (IκB), NF-kB may become constitutively active under the influence of cytokines such as IL-1β and this is found to be the case in many cancers.

Wang et al demonstrated a pro-tumourigenic role of interleukin 1β by using recombinant IL-1RA to block its actions in an in vivo mouse model of colitis-associated cancer. This resulted in reduced inflammatory cell infiltration and tumour development. Other studies have shown IL-1β to directly promote tumour cell proliferation and this is thought to be through activation of the Wnt-β catenin pathway.

Tumour Necrosis Factor α (TNFα)

Pro-inflammatory cytokine TNFα has been widely studied in the context of cancer-associated inflammation. Like IL-1β, it produces many of its carcinogenic and pro-tumour actions through activation of the oncogenic transcription factor NF-kB. Luo et al demonstrated this in their mouse model of colon adenocarcinoma, where lung metastasis growth was prevented by the inhibition of TNFα-mediated NF-kB activation.

In colorectal cancer, it is produced mainly by activated macrophages.
**Figure 8.** Actions of NF-kB (Nuclear factor KB) in tumour microenvironment.

*Interleukin 8 / CXCL8 (IL-8)*

IL-8 expression is frequently increased within CRC tumour tissue compared to healthy adjacent intestinal tissue in humans\(^2\)\(^0\)\(^2\). In inflamed tissues, IL-8 release is predominantly from myeloid cells after transcription is up-regulated by NF-kB\(^2\)\(^0\)\(^3\), which in turn is induced rapidly by TNFα and IL-1β, as discussed above\(^2\)\(^0\)\(^4\).

IL-8 is a potent chemo-attractant of granulocytes and, particularly, neutrophils to sites of inflammation\(^2\)\(^0\)\(^5\). CRC cell line studies have shown that IL-8 promotes tumour growth and angiogenesis by activation of Akt and mitogen-activated protein kinase (MAPK) signalling pathways\(^2\)\(^0\)\(^6\),\(^2\)\(^0\)\(^7\). Martin et al described the upregulation of VEGF by endothelial cells in response to IL8 via NF-kB, independent of hypoxia-inducible factor 1α (HIF-1α)\(^2\)\(^0\)\(^8\). This
study implicates IL-8 in a pro-angiogenic pathway that may occur in the absence of hypoxia.

Finally, IL-8 is also thought to contribute toward invasion and metastasis through epithelial-mesenchymal transition (EMT) and the expression of matrix metalloproteinases in endothelial cells 209, 210.

**Interleukin 6 (IL-6)**

Interleukin 6 is one of the most extensively studied cytokines in CRC as it plays a central role as a prototypic pro-inflammatory cytokine 211. Historically, macrophages have been thought to be the main source of IL-6 in the TME but CAFs are also thought to be a major contributor 212, 213. IL-6, along with its family members IL-11 and IL-27, play a central role in cancer progression as activators of oncogenic transcription factor pathway Janus kinase (JAK)- signal transducer and activator of transcription 3 (STAT3) 193, 214. The JAK/STAT3 pathway may also be activated by HGF and EGF 215.

Downstream actions of the IL-6/JAK/STAT3 pathway include tumour cell survival, proliferation, migration and angiogenesis 216. The latter is thought to be through increased VEGF-A transcription in response to HIF-1 217.

In addition to this, there is also evidence of an immunomodulatory role of IL-6. Tsukamoto et al demonstrated this in IL-6 deficient mice, where IL-6 produced by myeloid-derived suppressor cells limited the development of T_H1 cells, to promote tumour progression 218.
Tumour growth factor β (TGFβ)

The effects of TGFβ on tumour growth within the TME have been described as dualistic to the point of being named the molecular Jekyll and Hyde of cancer \(^{219}\). Studies have demonstrated the context-specific nature of TGFβ’s effects as initially tumour-suppressive in epithelial cells and then tumour-promoting in the later stages of cancer development \(^{219}\). The SMAD proteins one to nine (SMAD1-9) are effector proteins downstream of the interaction between members of the TGFβ superfamily and their receptors \(^{220}\). SMAD4 lies at the convergence point of these pathways and is found to be abnormally expressed in 20-30% of CRCs \(^{221}\). Experimental studies are hampered by most SMAD4 knockout mice dying in utero \(^{220}\) but Freeman et al reported a ten-fold increase in colonic adenomas in heterozygous mice \(^{222}\). These findings support the tumour suppressor role of TGFβ.

In contrast, colorectal cancer stem cells treated with TGFβ have been noted to increase their invasive and metastatic potential as well as their ability to promote angiogenesis and suppress adaptive T cell immune responses \(^{223}\). In their review, Yao et al describe a large body of evidence in support of TGFβ acting as an important regulator of EMT, endowing growing cancers with the potential to intravasate and therefore disseminate \(^{224}\).

Interferon γ (IFNγ)

Interferon gamma is produced primarily by T cell lymphocytes and natural killer cells and plays an important role in anti-tumour immunity \(^{225},^{226}\). Some of its main immunomodulatory effects include the activation of cytotoxic T cells, stimulating major histocompatibility- (MHC-)mediated antigen presentation pathways and inducing a T\(_H\)1
helper cell response\textsuperscript{227}. IFNγ also has a direct inhibitory effect on cancer growth and may induce cancer cell apoptosis via Bcl-2 downregulation\textsuperscript{228}.

\textit{Interleukin 10 (IL-10)}

Interleukin-10 is a potent anti-inflammatory cytokine produced by tumour cells in addition to a range of immune cells including M\textsubscript{2} macrophages, dendritic cells, B cells and T\textsubscript{H}\textsubscript{2} cells\textsuperscript{229}. Its signalling is through JAK/STAT pathways and functions predominantly as an immunoregulatory molecule\textsuperscript{230}. As IL-10 may inhibit both innate and adaptive immune processes it has been shown to exert a combination of both anti-tumour and pro-tumour downstream effects\textsuperscript{202,231}. Pro-tumour effects of IL-10 include inhibition of dendritic cell maturation and anti-tumour effects result from inhibition of NF-κB\textsuperscript{229}.

\textbf{Summary}

In addition to tumour cells, the cellular infiltrate within the tumour microenvironment is a mix of inflammatory cells of the innate and adaptive immune systems as well as stromal cells such as fibroblasts. Some of these cell types display polarisation toward sub-types which in turn have primarily pro- or anti-tumour actions and in the case of neutrophils and macrophages display a certain plasticity which allows them to ‘change sides’ depending on the biochemical landscape. Through the actions of cytokines, these cell-types have the ability to influence local processes such as tumour growth, angiogenesis and invasion as well as enlist haematopoietic and lympho-retticular cellular resources from the circulation. Many of the signalling pathways involved are overlapping, with oncogenic transcription factors NF-κB and STAT3 being central players, and cytokines may stimulate their own expression through autocrine or paracrine feedback loops.
Cancer-related Inflammation: Systemic Processes

Our understanding of the interaction between inflammation and cancer has been derived primarily from pathways acting within the local microenvironment, covered previously. Although a relatively young field, there is a growing body of evidence suggesting an important role for inflammation on cancer progression at the systemic level (Figure 9)\textsuperscript{232}. McMillan et al observed an association between raised pre-treatment C Reactive Protein (CRP) and poor prognosis in patients following curative surgery for colorectal cancer\textsuperscript{233} and consequently went on to develop the modified Glasgow Prognostic Score (mGPS)\textsuperscript{234}. The score, which combines CRP with albumin has since been validated in a number of cancers alongside colorectal\textsuperscript{235} with other scoring systems following suite, similarly based on indicators of systemic inflammation. Most notably, this includes the neutrophil-lymphocyte ratio\textsuperscript{236}. Despite the challenges in implementing experimental models of organism-wide effects of cancer-related inflammation, a number of mechanisms have been proposed.

Figure 9. Systemic pathways: Inflammation and cancer progression.
Neutrophil mobilisation

As discussed previously, tumour-derived chemokines act locally to attract inflammatory cells from the surrounding tissue. In addition to this, there is evidence that tumours release factors such as G-CSF into the circulation in order to mobilise circulating cells from distant reservoirs, particularly the bone marrow and spleen\textsuperscript{237, 238}. A raised circulating neutrophil count is frequently observed in patients with advanced colorectal cancer as well as other malignancies\textsuperscript{161} and mouse models have supported this relationship to be causal, where mice inoculated with tumour develop an acute neutrophilia\textsuperscript{173, 239}. In addition to the number of circulating neutrophils, the morphology has also been found to differ between cancer patients and healthy volunteers\textsuperscript{240}. Low density neutrophils (LDNs) have been described, that are scarcely found in healthy cases and found with increasing frequency in cancer cases in association with increasing tumour growth\textsuperscript{240}. The function of LDNs are thought to differ from high density neutrophils (HDNs). Granot et al’s series of experiments using a mouse model of breast cancer showed G-CSF-stimulated circulating neutrophils to possess cytotoxic ability and thus inhibited metastatic seeding\textsuperscript{239}. In contrast to this LDNs inhibited CD8+ cytotoxic T cell proliferation and exhibited reduced cytotoxicity toward cancer cells, allowing them to grow and divide\textsuperscript{161}. Whether LDNs or HDNs are mobilised into the circulation is thought to be determined by the specific cytokines released by the primary tumour. This hypothesis arose from studies such as that by Marini et al who found that G-CSF administration to healthy volunteers led to an immunosuppressive neutrophilia\textsuperscript{241}. 
Systemic Immunomodulation

In addition to the influence circulating neutrophils have on anti-cancer immune mechanisms, Regulatory T cells (T_{Reg}) play an important role in cancer-induced immunomodulation. T_{Reg} cells are a subset of CD4+ T cells that are necessary under physiological conditions to prevent auto-immunity. They are activated by TGFβ and IL-10 and inhibit anti-tumour immunity through direct action on CD8+ cytotoxic T cells and by preventing dendritic cell maturation^{242-244}. In their study using hepatocellular carcinoma cell lines, Yang et al demonstrated that Treg cells were attracted into the tumour microenvironment via a TGFβ-dependent pathway and that this was necessary for metastasis to occur in mice^{245}.

Endothelial activation

Endothelial activation has been observed in metastatic colorectal cancer patients, indicated by raised serum levels of soluble endothelial cell adhesion molecules, e-selectin and vascular cell adhesion molecule 1 (VCAM-1)^{246}. Endothelial activation is thought to promote metastasis by the expression of cell surface adhesion molecules that trap circulating tumour cells^{247}. Additionally, the release of chemokines by activated endothelial cells attract host inflammatory and immune cells to create a receptive microenvironment^{247}. Okahara et al’s study on a murine lung-metastasising model of melanoma found that inoculation of the mice with TNFα led to endothelial activation and increased metastasis formation^{248}. Together, these findings suggest that endothelial activation at distant sites may be a means through which primary tumour-derived cytokines promote metastasis.
**Platelet activation**

Thrombocytosis, a raised circulating platelet count, is frequently observed in patients with colorectal cancer compared to healthy controls and has been reported to increase in metastatic disease. The platelet count has also been noted to correlate with circulating cytokine levels, particularly interleukin-6. Kuznetsov et al’s study on mice carrying luminal breast cancer cell xenografts produced platelets that contained pro-angiogenic and pro-inflammatory cytokines including TGFβ1, VEGF and IL-6. Platelets have been implicated in other pro-metastatic pathways through direct contact and interaction with circulating tumour cells. This has been demonstrated through inducing the expression of IL-8, MMP-9 and VEGF, and by inducing endothelial-mesenchymal transition via TGF-β and NF-kB signalling.

**Pre-metastatic niche formation**

The ‘seed and soil’ theory was first proposed in 1889 by Stephen Paget, who examined post-mortem data from 735 women with breast cancer and determined metastases to be preferentially distributed toward favoured organs. Paget went on to hypothesise that both tumour cells with metastatic potential (the ‘seed’) and a receptive environment (the ‘soil’) at predetermined sites, were necessary to form metastatic deposits. Contemporary evidence has continued to support this theory and points to biochemical and cellular changes that occur within distant organs, leading to a supportive microenvironment, termed the ‘pre-metastatic niche’. Tumour-derived secreted factors as well as immune, inflammatory and stromal cells are thought to contribute to its formation.
In their important study, Kaplan et al demonstrated VEGFR+ bone marrow derived cells (BMDCs) 'homing' to distant organs prior to the arrival of tumour cells in mice \(^{256}\). They also found evidence that soluble factors derived from the primary tumour acted on fibroblasts at distant sites to upregulate growth factors that then contributed to a permissive microenvironment to incoming tumour cells \(^{256}\). Yamamura et al found fibroblasts in the pre-metastatic niche to secrete TGFβ and matrix metalloproteinases, which in turn are known to influence tumour growth and progression by acting in a paracrine manner \(^{258}\). Alterations in the local levels of inflammatory cytokines have also been reported to include a reduction in interferon gamma and increases in T\(_{H}2\) cytokines and MMP-9 in the pre-metastatic lung \(^{259}\). Rutowski et al described the mobilisation and accumulation of MDSCs (myeloid-derived suppressor cells) within the pre-metastatic organs of mice, which was found to be driven by IL-6 and IL17 \(^{260}\).

Neutrophils have been implicated in the formation of a pre-metastatic niche, particularly in the lungs. Wu et al carried out experiments on 4TI mammary carcinoma and Lewis lung carcinoma models in mice and found that a high neutrophil infiltration was observed in pre-metastatic lungs, a process thought to be mediated by G-CSF \(^{261,262}\). In addition to this, Wculek and Malanchi showed neutrophils to be a main component of tumour growth within the pre-metastatic lung in mouse breast cancer models \(^{263}\). Matrix metalloproteinase 9 is also thought to play an important role in inducing pro-tumourigenic effects of neutrophils within the pre-metastatic lung \(^{264}\).

Studies examining pre-metastatic niche formation using colorectal cancer models are relatively sparse. One such study is that by Seubert et al who demonstrated primary tumour-derived tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) was necessary for
hepatic metastasis of colon cancer cells. A study by Wang et al found that CXCL1 secreted from TAMs within the primary tumour microenvironment recruited MDSCs into the pre-metastatic liver in colorectal cancer, and that CXCL1 production was in turn stimulated by tumour-derived VEGF. These studies suggest that a pre-metastatic niche may develop in colorectal cancer and that tumour- and immune cell-derived soluble factors influence their formation. Additionally, Shao et al demonstrated tumour-derived small extracellular vesicles to specifically target the liver where a pro-inflammatory pre-metastatic niche was established through IL-6 secretion by Kupffer cells. The extracellular vesicles were found to be rich in micro RNA (miR-21), a marker that itself has been shown to correlate with prognosis.

_Circulating Tumour Cells_

Tumour cells that enter the systemic circulation through venous or lymphatic drainage of the tumour, known as circulating tumour cells (CTCs), are a necessary precursor to haematogenous metastases through seeding of distant organs. Two pathways of intravasation of tumour cells have been described, the first being through EMT whereby tumour cells de-differentiate before taking on a mesenchymal phenotype with increased invasive and migratory ability to enter established blood vessels. A second pathway that has been described more recently is through passive ‘tumour shedding’ of CTCs into neo-vascular structures. This is considered to occur at a much earlier stage in tumour progression, with some suggesting it to occur as early as the angiogenic switch, soon after a tumour has acquired invasiveness. This has led to interest in quantifying CTCs as a means of predicting early-stage metastases. Whilst CTCs, particularly when found in clusters, have shown some promise in predicting prognosis, there are some significant
disadvantages. The rarity and heterogeneity of CTCs has made accurate quantification technically challenging. Furthermore, less than 0.02% of CTCs are thought to result in a metastatic mass. This is due to inefficiencies of metastatic colonisation which is considered to be the rate-limiting step and influenced also by the permissiveness of the local microenvironment.

In their study using the B16F0 melanoma cell line in mice, Li et al showed that circulating tumour cells triggered a systemic inflammatory response (indicated by raised serum G-CSF and IL-6 levels), resulting in increased pro-metastatic neutrophil infiltration and MMP-9 expression within the lung. In their model, metastatic colonisation was inhibited by administration of anti-inflammatory cytokine, interleukin-37. Divella et al demonstrated this principle to translate to humans, by reporting circulating inflammatory cytokines TGFβ and CXCL1 levels to be significantly associated with the presence of clustered CTCs and worse survival in patients with metastatic colon cancer.

**Summary**

A number of mechanisms have been proposed linking systemic inflammation to CRC growth and progression. Cells within the primary microenvironment may enlist these mechanisms to facilitate their own growth or to produce alterations at distant sites that aid dissemination. There is also evidence that inflammatory changes occur even before the arrival of tumour cells at so-called pre-metastatic niches. Furthermore, interactions that occur between tumour-related cells within the circulation also influence the systemic immune and inflammatory environment and consequently may have a bearing on primary and metastatic progression.
1.3 Prognostic studies linking systemic inflammation to outcome in CRC

Basic science research has led to a growing appreciation of the interaction between the tumour and its host. In parallel to this, clinical studies have set-out to apply this principle by evaluating the relationship between systemic inflammation and prognosis. This has been driven in part by a need to better prognosticate and subsequently guide individualised treatment strategies. The TNM staging system is the current gold standard and has been applied in a form that has remained largely unchanged for decades. The system is based on a paradigm of linear metastatic progression from local invasion, through lymphatic spread, to distant metastasis that has recently been challenged. An alternative model termed the parallel progression model is now becoming increasingly recognised whereby distant metastasis is a potentially early event leading to deposits that then develop in parallel to that of the primary tumour. Study into systemic markers of cancer prognosis has accelerated in response to this proposed mechanism.

*C Reactive Protein and the Glasgow Prognostic Score*

The Glasgow prognostic score (GPS) has been studied in a number of cancers since it was first reported to predict poor prognosis in non-small-cell lung carcinoma by Forrest et al seventeen years ago. Combining pre-treatment serum levels of CRP and albumin, the score is based on the criteria shown in Table 6. Prior to its modified form, GPS originally included a score of ‘1’ for an isolated hypoalbuminaemia although this was subsequently found not to predict poor prognosis and therefore removed.
Table 6. Scoring criteria of modified glasgow prognostic score (mGPS).

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive protein ≤ 10 mg/L</td>
<td>0</td>
</tr>
<tr>
<td>C-Reactive protein &gt; 10 mg/L and albumin ≥ 35 g/L</td>
<td>1</td>
</tr>
<tr>
<td>C-Reactive protein &gt; 10 mg/L and albumin &lt; 35 g/L</td>
<td>2</td>
</tr>
</tbody>
</table>

McMillan et al performed a systematic review of the literature and reported 28 studies (n>8000) evaluating the prognostic value of GPS/mGPS in patients with operable cancer and found all 28 studies to demonstrate a statistically significant multivariate hazard ratio. The Glasgow group represents a dominant presence in this field, which in itself may have introduced bias into the review; eight out of twelve studies limited to patients with primary colorectal cancer were performed by their group. A further meta-analysis was performed more recently by Liu et al and focussed on the prognostic value of GPS in colorectal cancer. Including 25 studies (n=5660), they concluded that increased GPS was closely associated with overall survival and cancer-specific survival both in primary operable and advanced inoperable disease. Both systematic reviews highlighted interstudy heterogeneity and that a large proportion of studies were limited by a retrospective design. Whilst both review articles noted an association between GPS and TNM stage, they also reported the association of GPS with prognosis to remain present once TNM stage was controlled for.
Neutrophil lymphocyte ratio (NLR), Platelet lymphocyte ratio (PLR) & Lymphocyte monocyte ratio (LMR)

A number of indices based on inflammatory markers within the full blood count have been shown to yield some prognostic benefit in CRC\textsuperscript{280}. The most widely studied of these is the neutrophil-lymphocyte ratio (NLR), which was initially developed as a marker of sepsis severity in critically ill patients\textsuperscript{281}. Its application to cancer was thought to reflect a combination of increased pro-tumour systemic inflammation and a reduced anti-tumour adaptive immune response, indicated by neutrophilia and reduced lymphocyte count, respectively\textsuperscript{282}. Although the results of some studies have been equivocal particularly in predicting recurrence in rectal cancer\textsuperscript{283, 284}, overall, NLR is considered a reasonable prognostic indicator\textsuperscript{236}. On one hand, NLR has the advantage of being inexpensive and calculated from laboratory tests that are ordered routinely in the pre-operative setting. On the other hand, like the evidence for GPS, the majority of studies are also limited by their retrospective design.

Additional indices including the platelet-lymphocyte ratio (PLR) and lymphocyte-monocyte ratio (LMR) have since emerged as markers that also reflect inflammatory processes at the tumour level and systemically and show an association with outcome\textsuperscript{285}. Chan et al’s recent Australian study included 1623 patients that underwent curative resection\textsuperscript{286}. Although it was a retrospective analysis in which 954 patients were excluded due to missing data, its large sample size allowed sub-group analysis to be performed by stage. A significantly better overall survival was seen in patients with a high LMR by individual stage and, by comparison, the LMR was touted as a superior prognostic marker to the more established GPS and NLR\textsuperscript{286}.
A disadvantage shared by each of these indices is that the function of their cellular components show some plasticity and may be influenced by interactions with cytokines. As discussed previously, neutrophils for example take on a phenotype with predominantly pro- or anti-tumour effects depending on the milieu of cytokines they are exposed to. Therefore simply quantifying these cells is unlikely to yield optimal prognostic value. Likewise, CRP is an acute phase protein produced and released by hepatocytes in response to stimulation by pro-inflammatory cytokines, including IL-6. Attention therefore has turned appropriately upstream to individual circulating cytokines.

**Cytokines and prognosis in CRC**

Interleukin-6 is the most studied cytokine for its prognostic value in colorectal cancer. Two meta-analyses have been published on this topic and include a combined total of eleven unique studies. Both reviews concluded that pre-treatment circulating IL-6 levels were predictive of 5-year overall survival. However, neither review were conclusive as to whether IL-6 predicted recurrence. This was cited to be due to a lack of available data and heterogeneity between studies. Additionally, the sample sizes of the studies were small, with only three studies including more than 100 patients.

More recently, a study by Shiga et al evaluated the ability of pre-treatment IL-6 to predict recurrence in 207 CRC patients undergoing curative intent resection. The study found IL-6 to be an independent predictor of recurrence and found this to be the case for TNM stage II (n=60) patients in sub-group analysis (p=0.01). As a prospective study that was sufficiently powered to include sub-group analysis of recurrence in stage II patients the study by Shiga et al was well-designed, although further clinical study is necessary to validate these findings.
In addition to IL-6, a wide spectrum of cytokines have been shown to correlate with prognosis in colorectal cancer. These include pro-inflammatory cytokines (IL-1β, TNFα, TGF-β), anti-inflammatory cytokines (IL-10, IFNγ), chemokines (IL-8, CXCL1), pro-angiogenic factors (VEGF), proteinases (MMP-9) and factors that stimulate haematopoietic cell production (M-CSF). Overall, the studies are limited by small sample sizes and a high degree of inter-study heterogeneity. A systematic review of prognostic studies evaluating multiple cytokines is included in Chapter Three of this thesis.

1.4 Shortfalls of the TNM staging system

The TNM staging system is currently the best known and most widely implemented method of predicting prognosis in CRC. However, prognostic biomarker research in CRC is fuelled by growing criticism of the TNM paradigm.

At its foundation, the TNM staging system remains tied to the archaic Dukes’ classification which implies a sequential progression from local invasion through regional lymph node involvement to distant metastasis. Anatomically, metastasising tumour cells may reach the systemic circulation and hence distant organs not only by lymphatic spread (via the thoracic duct) but also by haematogenous means. This gives rise to the potential bypassing of the regional lymph nodes. Although lymph node involvement may be considered a surrogate marker for more advanced disease a growing body of evidence challenges this premise by supporting what has been termed the ‘parallel invasion model’.

Here, metastases arise from tumour cells that disseminate early in the development of the primary tumour; distant metastases then progress in parallel to the primary tumour. This model is supported by genetic studies such as that by Naxerova et al where corresponding samples from the primary tumour, regional lymph
nodes and distant metastases from individual patients were found to be genetically distinct\textsuperscript{276}.

A further flaw of the TNM staging system in colorectal cancer is the heterogeneity of outcomes in patients of the same TNM stage \textsuperscript{314}. For instance, according to analyses from the Surveillance, Epidemiology, and End Results (SEER) database, 5-year survival in patients with stage II colon cancer range from 87.5-58.4%, depending on T stage\textsuperscript{315}. Additionally, 5-year survival rates in patients with stage III ranged from 90.7% to 15.7% depending on the T stage and N stage\textsuperscript{315}. Similarly wide ranges were seen amongst stage II and III rectal cancer with a large overlap seen between them\textsuperscript{315}. The SEER database is a population-based database that represents 26% of the United States and is maintained by quality assurance studies that set a standard of 98% accuracy\textsuperscript{316}. Whilst this does make it a robust resource, results may also be confounded by stage II patients receiving less aggressive therapy and comprising a higher proportion of patients with inadequate lymph node sampling\textsuperscript{317}. Additionally it is noteworthy that the SEER database collects data on survival and not recurrence. However, the so-called “survival paradox” between stage II and III colorectal cancer has been further demonstrated in studies that have set out to address these issues\textsuperscript{318,319}. For example, Kim et al’s study of 5,547 South Korean colorectal cancer patients demonstrated a worse overall survival (p=0.012) and disease free survival (p=0.001) in patient with IIB/C disease compared to those with IIIA despite an even distribution of adjuvant chemotherapy and lymph node sampling between the groups\textsuperscript{319}. 
1.5 Aims

The overall aim of this thesis was to investigate the relationship between circulating inflammatory cytokine levels and prognosis in patients undergoing treatment of colorectal adenocarcinoma with curative intent. In contrast to more established prognostic factors, many of which are based on tumour extent and biology, our approach seeks to evaluate the host-response and its influence particularly on disease recurrence. This in turn may have implications for patient selection for different treatment and follow up strategies, whether curative or palliative; aggressive or conservative; intensive or standard surveillance.

The specific aims of this thesis were:

- To determine the clinical and pathological predictors of disease free survival and overall survival in a local population of patients suitable for treatment with curative at our Institution
- To perform a systematic review of the literature evaluating the prognostic value of cytokine analysis in colorectal cancer utilising a multi-marker approach
- To compare baseline levels of plasma IL-6, IL-1β, TNFα and IL-8 between three groups: healthy control, stage II and stage IV CRC and to compare cytokine profiles with C-reactive protein (CRP) and carcinoembryonic antigen (CEA)
- To perform a pilot study of baseline plasma IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, IFNY and TNFα as predictors of disease free survival in patients undergoing treatment of colorectal cancer with curative intent
Chapter Two
Retrospective Cohort Study of Prognostic Predictors following Treatment with Curative Intent
2.1 Introduction

Disease recurrence represents a failure of treatment with curative intent. Therefore, understanding the timing, location and risk factors of recurrence is essential to guiding appropriate treatment. For example, adjuvant chemotherapeutic agents, with their inherent risks and side effects, are likely to be of limited value in patients at already low-risk of recurrence. Furthermore, the incidence of disease recurrence among a patient group can be considered a measure of size of the clinical problem. A detailed evaluation of the patterns of recurrence may also provide insights into the mechanisms by which they occur, in the context of current clinical practice. Additionally, an analysis of the cohort characteristics and outcomes at our Institution allow comparison against International standards which, in turn, is necessary to establish the validity of this and future studies based at our Institution.

As outlined in Chapter One, a number of clinicopathological factors are considered established predictors of prognosis. These include, emergency presentation, TNM stage, high histological grade, mucinous cell-type, lymphovascular invasion, peri-neural invasion and pre-operative CEA. Furthermore, CEA is a widely available circulating biomarker, whose prognostic value in colorectal cancer is supported by a time-tested body of literature. Therefore, assessing pre-operative CEA as a prognostic biomarker in a local cohort study is an important step toward establishing it as a comparator against which to test other candidate markers.
Aims

The aims of this chapter are:

• to determine the characteristics of a local population of patients suitable for treatment with curative at our Institution, including:
  • the proportion of patients presenting in each TNM stage
  • the pattern of disease recurrence in terms of timing and location
  • the clinical and pathological predictors of disease free survival and overall survival
  • to evaluate pre-operative CEA as an independent predictor of disease free survival and overall survival
2.2 Methods

Patient selection

Eligibility criteria:

- Consecutive patients over the age of 18 years undergoing surgery with curative intent for newly diagnosed primary adenocarcinoma of the colon or rectum between January 2010 and December 2012 at our local institution were eligible for this study.

Exclusion criteria:

- Patients with a personal history of colorectal malignancy,
- Patients with stage IV disease at presentation that had residual disease following definitive treatment (R1 or R2)
- Patients in whom post-operative follow-up and surveillance was performed at another institution
- Patients with a complete pathological response to neoadjuvant therapy

Data collection

Patients were captured by performing a search of the records of operating theatre cases, histopathology specimens and hospital discharges. Data was collected from electronic hospital records, which included: operation notes, clinic letters, multi-disciplinary team meeting discussions, radiology reports and laboratory and histopathological results. The site of primary cancer was taken from the operation note and ‘disease stage’ was assigned in accordance with the American Joint Committee on Cancer (AJCC) Seventh edition.5
Where available, pathological staging was taken over clinical staging and those with complete pathological response were classified as Stage I.

**Surgery**

Surgery for rectal cancer was typically carried out by three colorectal surgeons and colon cancers by general and colorectal surgeons at the hospital.

**Follow Up Protocol**

A standardised protocol for post-operative follow-up and surveillance was used during the study period and included three to six monthly surgical outpatient visits and yearly computed tomography (CT) scans of the chest, abdomen and pelvis for the initial three years. A complete colonoscopy was performed within a year of surgery if one had not been performed pre-operatively with a repeat colonoscopy after three years. Yearly outpatient appointments were scheduled between years three to five and three monthly serum carcinoembryonic antigen (CEA) was checked for all five years.

**Assay**

Serum carcinoembryonic antigen levels were measured at the Wellington Hospital clinical laboratory by electrochemiluminescence using the commercially-available Roche Cobas 6000 analyser and the e601 assay, with a detection limit range of 0.200-1000nG/mL.

**End-points**

The primary endpoint studied was Disease free survival (DFS), defined as the time from surgery to recurrence or death. Secondarily, Overall survival (OS) was studied and
represented the duration from time of surgery to all-cause mortality. Patients that were alive and without evidence of disease recurrence on 1st November 2016 were censored.

**Statistical analysis**

Statistical analysis was performed using IBM SPSS Statistics Version 24.0. For continuous variables, a distribution with a p-value of greater than 0.05 found on Shapiro-Wilk testing was considered normal. Means were expressed with standard deviation for variables demonstrating a normal distribution and medians were expressed with interquartile range for those not demonstrating a normal distribution. \(X_2\) test and Fischer’s exact test were used for comparisons of categorical variables and Student t-test and Mann-Whitney U-test for continuous parametric and non-parametric variables, respectively.

A Receiver Operator Characteristics (ROC) curve was used to estimate the optimal cut-off value for CEA, using DFS as the endpoint of interest. The optimal cut-off corresponded to the point closest to 1.0 sensitivity -0.0 specificity on the ROC curve. The cohort was then divided into ‘High CEA’ and ‘Low CEA’ groups using this cut-off point.

Kaplan-Meier curves were plotted to evaluate DFS and OS and the Mantel Cox log-rank test performed to compare groups. Univariate and multi-variate survival analyses were performed using Cox proportional hazards model including variables with p<0.1 on univariate analysis into a multi-variate model optimised through the backward step-wise elimination method. The null hypothesis was rejected when p values were equal to or below 0.05.
Ethics and governance

This study complied with regulations for audit at the host institution and met the New Zealand definition of observation research. National ethics committee approval was not required for this study.

2.3 Results – All Patients

Patient and tumour characteristics

Two hundred and thirty seven patients were included in the study including 116 males and 121 females with a median age of 71 years (range 32 to 91 years). Two hundred and nine patients (88.2%) were European, 10 (4.2%) of Māori and nine (3.8%) of Pacific Island ethnicity. Fifty-nine (24.5%) cancers were rectal, 21 (8.7%) recto-sigmoid and the remaining 157 (66.2%) colonic. Thirty-six (15.2%) patients required an emergency operation which was most commonly due to obstruction (Table 7).
Table 7. Clinicopathological data of patients undergoing treatment of colorectal cancer with curative intent.

<table>
<thead>
<tr>
<th></th>
<th>n=237 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years (Range)</strong></td>
<td>71 (32 – 91)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>116 (48.9)</td>
</tr>
<tr>
<td>Female</td>
<td>121 (51.1)</td>
</tr>
<tr>
<td><strong>Co-Morbidities</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>156 (65.8)</td>
</tr>
<tr>
<td>1</td>
<td>63 (26.6)</td>
</tr>
<tr>
<td>2+</td>
<td>18 (7.6)</td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>59 (24.5)</td>
</tr>
<tr>
<td>Colon</td>
<td>178 (75.1)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>39 (16.5)</td>
</tr>
<tr>
<td>II</td>
<td>90 (38.0)</td>
</tr>
<tr>
<td>III</td>
<td>98 (41.4)</td>
</tr>
<tr>
<td>IV</td>
<td>10 (4.2)</td>
</tr>
<tr>
<td><strong>Emergency</strong></td>
<td></td>
</tr>
<tr>
<td>Emergency</td>
<td>36 (15.2)</td>
</tr>
<tr>
<td>Obstruction</td>
<td>26 (11.0)</td>
</tr>
<tr>
<td>Perforation</td>
<td>10 (4.2)</td>
</tr>
</tbody>
</table>
**Treatment undertaken**

**Surgery**

The operations performed for primary colorectal cancer were, right hemicolectomy or extended right hemicolectomy (n=97); transverse colectomy (n=3); left hemicolectomy (n=9); anterior resection (n=81); Hartmann’s procedure (n=15); abdominal perineal resection (APR) (n=20); subtotal colectomy (n=11); proctocolectomy (n=2); transanal excision (n=1).

Of the ten patients with stage IV disease at presentation, one had an oophorectomy for an ovarian metastasis at the time of initial surgery. The remaining nine had surgery for synchronous metastases following an interval (range 98-215 days) after the initial surgery.

Six patients underwent segmental liver resection, two patients underwent hemi-hepatectomy and one underwent an open excision of a lingular mass of the lung.

**Pre-operative and post-operative treatment**

Of 59 patients with rectal cancer, 39 (66.1%) underwent neo-adjuvant therapy. Fifteen (25.4%) of these received short course radiotherapy and 24 (40.7%) long course chemotherapy. Seventy-seven (32.5%) patients of the full cohort received adjuvant chemotherapy: 2.6% for stage I, 18.2% for stage II, 72.7% for stage III and 100% for stage IV.

**Recurrences**

The median follow-up duration was 61 months (46-81 months) and overall survival rate was 68.6%. In total, 59 (24.9%) patients developed disease recurrence, at a median time of 14.0 months.
Timing of recurrence

The annual recurrence rates were 9.7% for year one, 7.2% for year two, 3.8% in year three, 2.1% in year four and 0.8% for year five and greater (Figure 10). The median time to local recurrence was 12.5 months (IQR 8.5-26.0) with a similar time for distant recurrence at 14.0 months (IQR 8.0-25.0).

Site of recurrence

The most common site of disease recurrence was the liver (n=25) followed by the lung (n=16) and local (n=15) recurrence in colorectal cancer patients (Figure 11).
The overall recurrence rate was 26.4% for colon compared with 20.3% for rectal cancer (p=0.35); liver recurrences occurred in 11.8% of colon and 6.8% of rectal cancers (p=0.28); lung recurrences in 5.6% of colon and 10.2% of rectal cancers (p=0.23) and local recurrence occurred in 7.9% of colon cancer and 1.7% of rectal cancer (p=0.09).

Recurrences are summarised by stage in Table 8.

Figure 11. Site of recurrence by site of primary cancer.
Table 8. Recurrences (local and distant) by stage and site of primary.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Colon (%)</th>
<th>Rectum (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>21 (0)</td>
<td>18 (0)</td>
<td>39 (0)</td>
</tr>
<tr>
<td>Local</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Distant</td>
<td>0 (0)</td>
<td>2 (11.1)</td>
<td>2 (5.1)</td>
</tr>
<tr>
<td>Stage II</td>
<td>73 (10.9)</td>
<td>17 (5.9)</td>
<td>90 (10.0)</td>
</tr>
<tr>
<td>Local</td>
<td>8 (10.9)</td>
<td>1 (5.9)</td>
<td>9 (10.0)</td>
</tr>
<tr>
<td>Distant</td>
<td>7 (9.59)</td>
<td>4 (23.5)</td>
<td>11 (12.2)</td>
</tr>
<tr>
<td>Stage III</td>
<td>77 (7.8)</td>
<td>21 (6.1)</td>
<td>98 (6.1)</td>
</tr>
<tr>
<td>Local</td>
<td>6 (7.8)</td>
<td>0 (0)</td>
<td>6 (6.1)</td>
</tr>
<tr>
<td>Distant</td>
<td>21 (27.3)</td>
<td>4 (22.2)</td>
<td>25 (25.5)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>7 (0)</td>
<td>3 (0)</td>
<td>10 (0)</td>
</tr>
<tr>
<td>Local</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Distant</td>
<td>5 (71.4)</td>
<td>1 (33.3)</td>
<td>6 (60.0)</td>
</tr>
</tbody>
</table>

Disease Free Survival – All Patients

In Figure 12, Kaplan-Meier curves show a pronounced decrease in disease free survival in the first 24 months, across all four stages. The 5-year DFS by stage were 84.6 % in stage I, 64.4 % in stage II, 53.1 % in stage III and 30 % in stage IV.

On univariate analysis, emergency presentation, advanced TNM stage, histological high grade, lympho-vascular invasion and peri-neural invasion were significantly associated with DFS. Following multi-variate analysis, TNM stage and perineural invasion independently predicted disease free survival (Table 9).
Figure 12. Kaplan-Meier curves for disease free survival by disease stage (p=0.002).
Table 9. Univariate and multivariate analysis of predictors of disease free survival

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Emergency</td>
<td>1.63 (0.98-2.70)</td>
<td>0.06</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2.60 (1.09-6.21)</td>
<td>0.03</td>
</tr>
<tr>
<td>III</td>
<td>3.74 (1.60-8.78)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IV</td>
<td>6.00 (2.01-17.88)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mucinous</td>
<td>1.09 (0.61-1.97)</td>
<td>0.77</td>
</tr>
<tr>
<td>High grade</td>
<td>1.69 (1.03-2.77)</td>
<td>0.04</td>
</tr>
<tr>
<td>≥12 LN Yield</td>
<td>1.09 (0.72-1.64)</td>
<td>0.70</td>
</tr>
<tr>
<td>LVI</td>
<td>1.69 (1.11-2.58)</td>
<td>0.02</td>
</tr>
<tr>
<td>PNI</td>
<td>2.21 (1.34-3.65)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

LN: Lymph node; LVI: Lymphovascular invasion; PNI: Perineural invasion

Overall Survival – All Patients

There were 74 deaths from all causes giving a 69.8% 5-year overall survival for the cohort.

The overall survival by stage were, 87.2% in stage I, 76.7% in stage II, 58.2% in stage III and 30.0% in stage IV. The Kaplan-Meier curves in Figure 13 show that a decline in survival occurs after 36 months, particularly in patients with stage IV disease. Stage was the only factor on multivariate analysis to be associated with overall survival (Table 10).
Figure 13. Kaplan-Meier curves for Overall Survival by disease stage (p<0.001)
Table 10. Univariate and multivariate analysis of predictors of overall survival

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td><strong>Emergency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1.87 (0.71-4.96)</td>
<td>0.21</td>
</tr>
<tr>
<td>III</td>
<td>3.78 (1.49-9.56)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IV</td>
<td>6.19 (1.96-19.52)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Mucinous</strong></td>
<td>1.19 (0.66-2.17)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>High grade</strong></td>
<td>1.90 (1.12-3.24)</td>
<td>0.02</td>
</tr>
<tr>
<td>≥12 LN Yield</td>
<td>1.05 (0.66-1.67)</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>LVI</strong></td>
<td>1.48 (0.92-2.37)</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>PNI</strong></td>
<td>2.06 (1.18-3.59)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

LN: Lymph node; LVI: Lymphovascular invasion; PNI: Perineural invasion

2.4 Results – Pre-Operative CEA

Seventy-three patients were excluded as they underwent palliative treatment. Of these, 10 patients were co-morbid with locally advanced disease and 63 patients had widespread metastasis (Figure 14).

Out of 237 patients who underwent surgery with curative intent during the study period, 139 (58.6%) patients had preoperative CEA measured (Figure 14). This group had a median age of 71 years (range 32 to 91 years), 67 (48.2%) were male, 72 (51.8%) female, and 22 (15.8%) patients had stage I, 55 (39.6%) stage II, 58 (41.7%) stage III and 4 (2.9%) patients
had stage IV disease. The overall survival was 70.5%, and disease-free survival 61.6% at a median follow-up duration of 61 months (range 46-81 months).

Figure 14. Flowchart demonstrating patient numbers by CEA availability and primary site

**CEA Availability**

Baseline demographics and disease stage were compared between patients with missing (n=98) and available (n=139) pre-treatment CEA measurements, to evaluate potential selection bias. The two groups did not differ significantly by age, sex, comorbidities, site or stage of cancer (Table 11). A higher proportion of patients with missing CEA data had a history of smoking (p=0.02) and underwent emergency surgery (p=0.003) and Kaplan-
Meier curves did not demonstrate a significant difference between the groups for disease-free survival (Figure 15) or overall survival (Figure 16).

Table 11. Clinico-pathological data for patients by pre-treatment CEA availability.

<table>
<thead>
<tr>
<th></th>
<th>CEA Missing N=98</th>
<th>CEA Available N=139</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>71 (64-77)</td>
<td>71 (63-78)</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>49 (50.0)</td>
<td>67 (48.2)</td>
<td>0.79</td>
</tr>
<tr>
<td>Female</td>
<td>49 (50.0)</td>
<td>72 (51.8)</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Morbidities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>57 (58.2)</td>
<td>89 (64.0)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24 (24.5)</td>
<td>30 (21.6)</td>
<td>0.65</td>
</tr>
<tr>
<td>2+</td>
<td>17 (17.3)</td>
<td>20 (14.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>20 (20.4)</td>
<td>39 (28.1)</td>
<td>0.18</td>
</tr>
<tr>
<td>Colon</td>
<td>78 (79.6)</td>
<td>100 (71.9)</td>
<td>0.18</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>I</td>
<td>17 (17.3)</td>
<td>22 (15.8)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>35 (35.7)</td>
<td>55 (39.6)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>40 (40.8)</td>
<td>58 (41.7)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>6 (6.1)</td>
<td>4 (2.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Emergency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obstruction</td>
<td>17 (17.3)</td>
<td>9 (6.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Perforation</td>
<td>6 (6.1)</td>
<td>4 (2.9)</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Figure 15. Kaplan-Meier survival curves for disease free survival of patients by pre-treatment CEA availability (p=0.72).

Figure 16. Kaplan-Meier survival curves for overall survival of patients by pre-treatment CEA availability (p=0.66).
Optimum cut-off value

The median CEA value was 3.5ng/mL (range 0.4-2285ng/mL). A receiver operator curve was plotted for CEA against disease free survival and from this an optimum cut-off value of 3.0 ng/mL was chosen (sensitivity 0.70, specificity 0.52, area under the curve=0.62, p=0.02) which was then used to divide the cohort into two groups, ‘High CEA’ (n=73) and ‘Low CEA’ (n=65) (Figure 17).

There were no significant differences in baseline demographics between these groups although when pathological characteristics were compared (Table 12), the groups varied by site (p=0.05) and stage (p=0.03).

Figure 17. Receiver Operator Curve analysis of the ability of pre-treatment CEA to predict disease free survival. The black dotted line corresponds with the optimum cut-off value of 3.0 ng/mL.
<table>
<thead>
<tr>
<th></th>
<th>CEA Low N=56</th>
<th>CEA High N=82</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years (Median, IQR)</strong></td>
<td>67.5 (63.0-75.5)</td>
<td>73.0 (64.0-79.0)</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (48.2)</td>
<td>40 (48.8)</td>
<td>0.95</td>
</tr>
<tr>
<td>Female</td>
<td>29 (51.8)</td>
<td>42 (51.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Morbidities</strong></td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>0</td>
<td>40 (71.4)</td>
<td>49 (59.8)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9 (16.1)</td>
<td>20 (24.4)</td>
<td></td>
</tr>
<tr>
<td>2+</td>
<td>7 (12.5)</td>
<td>13 (15.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Rectum</td>
<td>21 (37.5)</td>
<td>18 (22.0)</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>35 (62.5)</td>
<td>64 (78.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>I</td>
<td>14 (25.0)</td>
<td>8 (9.8)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>21 (37.5)</td>
<td>34 (41.5)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>18 (32.1)</td>
<td>39 (47.6)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3 (5.4)</td>
<td>1 (1.2)</td>
<td></td>
</tr>
<tr>
<td><strong>Emergency</strong></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Obstruction</td>
<td>2 (3.6)</td>
<td>7 (8.5)</td>
<td></td>
</tr>
<tr>
<td>Perforation</td>
<td>1 (1.8)</td>
<td>3 (3.7)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Out of 38 patients with rectal cancer, 26 (68.4%) received neoadjuvant therapy which was in the form of short course radiotherapy in 10 (26.3%) and long course chemo-radiation in 16 (42.1%). Forty-three (30.9%) colorectal cancer patients received adjuvant chemotherapy which most frequently included Capecitabine alone (15.8%), followed by Capecitabine and Oxaliplatin (5.0%) and Folinic acid, fluorouracil and oxaliplatin in combination (3.6%). Use of neo-adjuvant therapy (p=0.33), adjuvant therapy (p=0.51), and
in-hospital morbidity (p=0.86) and in-hospital mortality (p=0.18) did not differ significantly between patients with high and low CEA.

Patient outcomes – Pre-operative CEA

Kaplan-Meier curves demonstrate that disease free survival (p=0.01) and overall survival (p=0.005) were significantly poorer in the ‘high CEA’ group (Figures 18 and 19, respectively).

Figure 18. Kaplan Meier curves of disease free survival by pre-treatment CEA (p=0.01)
On multivariate analysis, a high CEA (HR 1.99, 95% CI 1.07-3.69, p=0.03) and perineural invasion (HR 2.74, 95% CI 1.39-5.40, p=0.004) were identified as independent predictors of disease free survival (Table 13).
Table 13. Univariate and multivariate analyses for predictors of disease free survival.

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Emergency</td>
<td>1.93 (0.91-4.09)</td>
<td>0.09</td>
</tr>
<tr>
<td>Stage</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1.70 (0.64-4.57)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2.53 (0.98-6.56)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2.54 (0.49-13.1)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>1.03 (0.49-2.19)</td>
<td>0.93</td>
</tr>
<tr>
<td>High grade</td>
<td>1.50 (0.77-2.92)</td>
<td>0.23</td>
</tr>
<tr>
<td>≥12 LN Yield</td>
<td>1.13 (0.66-1.93)</td>
<td>0.67</td>
</tr>
<tr>
<td>LVI</td>
<td>1.73 (1.00-3.00)</td>
<td>0.05</td>
</tr>
<tr>
<td>PNI</td>
<td>3.07 (1.57-6.00)</td>
<td>0.001</td>
</tr>
<tr>
<td>Raised CEA</td>
<td>2.20 (1.20-4.06)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

LN: Lymph node; LVI: Lymphovascular invasion; PNI: Perineural invasion
On multivariate analysis for overall survival, a high CEA was identified as the only independent predictor of poorer disease free survival (HR 3.17, 95% CI 1.46-6.89, p=0.004) (Table 14).

Table 14. Univariate and multivariate analyses for predictors of overall survival.

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Emergency</td>
<td>2.26 (1.00-5.10)</td>
<td>0.05</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1.12 (0.39-3.76)</td>
<td>0.14</td>
</tr>
<tr>
<td>III</td>
<td>2.42 (0.84-6.99)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2.52 (0.46-13.78)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>1.50 (0.69-3.26)</td>
<td>0.30</td>
</tr>
<tr>
<td>High grade</td>
<td>1.65 (0.79-3.46)</td>
<td>0.18</td>
</tr>
<tr>
<td>≥12 LN Yield</td>
<td>1.05 (0.57-1.94)</td>
<td>0.88</td>
</tr>
<tr>
<td>LVI</td>
<td>1.31 (0.69-2.50)</td>
<td>0.42</td>
</tr>
<tr>
<td>PNI</td>
<td>2.01 (0.89-4.55)</td>
<td>0.09</td>
</tr>
<tr>
<td>Raised CEA</td>
<td>3.17 (1.46-6.89)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

LN: Lymph node; LVI: Lymphovascular invasion; PNI: Perineural invasion
2.5 Discussion

In this study, a recurrence rate of 24.9% was found at 5 years, with a median time-to-recurrence of 14 months and mortality rate of 31.2%; these findings are comparable to the modern International literature. Recurrence most commonly occurred in the liver, followed by lung, with a local recurrence rate of 6.3%. Recurrence was strongly linked to disease stage. Stage II patients made up 37.9% of all those undergoing surgery with curative intent and had a recurrence rate of 22.2%. Recurrences in stage II patients made up 33.9% of all recurrences observed.

Twenty-five out of 59 (42.4%) recurrences were observed in the first year following surgery and 43 (72.9%) within the first two years, with annual recurrence rates decreasing sharply thereafter. That the majority of recurrences occur within the first two years post-operatively is reflected in several guidelines which recommend increased frequency of follow-up clinic appointments within the first two to three years, such as those of the American Cancer Society, National Comprehensive Cancer Network and European Society for Medical Oncology. Studies with longer formal follow-up programmes have demonstrated low recurrence rates beyond five years also and this includes Seo et al’s study of 4,023 patients that revealed 36 (0.9%) recurrences occurred beyond five years.

Our results are consistent with others in demonstrating the liver as the most common site of disease recurrence for rectal and colonic primaries combined. Recurrences in the lung were relatively more common for rectal primaries compared with colonic, occurring in 10.2% and 5.6% respectively, although this difference was not statistically significant (p=0.28). This pattern is frequently attributed to systemic venous drainage of rectal cancers via the pelvic veins. Although much less common, we found
other sites of disease recurrence to include bone, brain, distant lymph nodes, peritoneum and ovary. Whilst we did not observe any recurrences in the spleen, this has also been reported previously, albeit infrequently.\textsuperscript{336}

We found recurrence rates to range from 5.1\% to 60\% in stages I to IV and multi-variate analysis identified disease stage as a strong independent predictor of overall and disease free survival.

The all-cause mortality rate in this cohort of 31.4\% compares favourably to rates of approximately 40\% reported historically in New Zealand and suggests a continuing trend of improving outcome.\textsuperscript{337-339} Data from the SEER database reveals a five year mortality of 35.1\% although this is not limited to patients undergoing surgery with curative intent.\textsuperscript{14}

The proportion of patients receiving adjuvant therapy in this cohort, 32.5\%, falls short of the 40\% reported by Buchwald et al, who have demonstrated a steady increase in the use of adjuvant therapy at their tertiary centre between 1993 to 2009.\textsuperscript{340} The proportion of stage III colon cancer patients receiving adjuvant chemotherapy was 58.4\% in this cohort, which is very similar to 59\% reported in the Presentations, Investigations, Pathways, Evaluation and Rx (PIPER) project report. Likewise, 34.7\% of patients with non-metastatic rectal cancer in this cohort compared with 36\% in the PIPER project report received adjuvant chemotherapy.\textsuperscript{341} For rectal cancer, 66.1\% of patients received some form of neo-adjuvant therapy which falls between the national average of 52\% reported in the PIPER project report and 82\% achieved in the 2009 cohort reported by Buchwald et al.\textsuperscript{340, 341} This demonstrates variability in the use of neo-adjuvant treatment, some of which may represent access to resources. However, the figures presented in this cohort are in concordance with other national datasets.
We report a very low rate of local recurrence following rectal cancer surgery of 1.7% and no patients had both local and distant recurrence. This compares well against modern international studies, where rates of 2.4-10% are frequently reported \(^{327}\). Rectal cancer surgery at the unit is performed exclusively by surgeons with subspecialist training and may go toward explaining the low local recurrence rates and indeed the one case of local recurrence was in a patient that needed an APR and declined surgery for 15 months. This also reflects a large improvement from rates in excess of 20% reported in the literature prior to the introduction of total mesorectal excision, adjuvant therapies, multidisciplinary team discussion and surgical subspecialisation \(^{338, 342}\).

Although the findings of this study were not novel to the field of Colorectal Surgery, the outcomes observed in this retrospective cohort study were comparable to those reported in the contemporaneous surgical literature. This helps to establish the study population as appropriate for further prognostic study.

Prognostic Value of Pre-Operative CEA

We found that 58.6% of patients had a pre-treatment CEA measured and this was more likely to be performed in the elective setting compared to emergencies. Given the high proportion of missing data, pre-operative CEA was excluded as a co-variate in survival analyses of the entire cohort. Using ROC curve analysis to select an optimal cut-off value, we found that a raised pre-treatment CEA was an independent predictor of disease free and overall survival after a median follow-up period of 61 months. Our observation that CEA was an independent predictor of survival is consistent with the findings of other studies \(^{130, 343-347}\). Becerra et al analysed data from 137,381 patients from a USA-based national database and found a raised pre-treatment CEA to be predictive of all-cause
mortality (HR 1.62, 95% CI 1.53-1.74, p<0.0001) independent of stage in stage I to III colon cancer. Tarantino et al demonstrated pre-treatment CEA to be predictive of overall survival (HR 1.46, 95% CI=1.02-2.08, p=0.044) and cancer-specific survival (HR 3.28, 95% CI=1.78-6.03, p<0.001) in 1932 patients with stage I rectal cancer. Additionally, Kim et al found pre-treatment CEA to predict disease free survival and overall survival in patients with stage III colon cancer following curative resection with adjuvant chemotherapy.

Whilst these large studies suggest a prognostic role for CEA within subgroups of colorectal cancer patients at a population level, our study reveals an effect that is observable across disease site and stage at an individual level and this may be useful in informing personalised treatment decisions.

A number of methods have been utilised to select a cut-off value for CEA in the literature. Although seemingly arbitrary, 5ng/mL has been used by many groups, almost as convention. Other investigators have used reference ranges given by their Institution’s laboratory and others still have determined optimal cut-off values based on ROC curves, as was implemented in the present study. It is noteworthy that the cut-off value of 3.0 ng/mL selected in this study is lower than the 5ng/mL value proposed by AJCC and that conventionally used in the literature. Individualised cut-off values have been used by Reiter et al, in which 4ng/mL was taken, and by Jeon et al, who identified 5.5, 4.8 and 3.5ng/mL as optimum cut-off values for TNM stages I, II and III, respectively. Kim et al found a CEA above 3.0ng/mL independently predicted DFS and OS in stage III colon cancer. Taken together, these results suggest that a relationship between pre-operative CEA and prognosis does exist, but that its implementation in clinical practice is hampered by the likely disease- and stage-specific nature of the effective cut-off value.
Limitations

The current study has a number of limitations to acknowledge.

From the outset, continuous variables, such as pre-operative CEA, were dichotomised. This approach was selected in order to allow simple risk-stratification and to produce results that could be compared against existing studies, many of which have taken the same approach. However, it must be noted that potentially valuable prognostic information may be lost by simply stratifying the cohort into “high” or “low”. An alternative approach would be to perform linear regression, although this assumes a linear relationship between variable and outcome. Additionally, unless the median is taken as the cut-off point, invariably the two groups become unequal in size. In the case of the present study, 82/138 (59.4%) had a raised CEA. In practical terms, a CEA of less than 3.0ng/mL would be more clinically useful in identifying a low-risk population than treating all patients with a raised CEA as high-risk, which may in turn result in over-investigation or –treatment.

A further limitation to the study is in determining an optimal cut-off value for pre-operative CEA based on the same sample that is being studied. This method has the potential to over-estimate the effect of CEA on prognosis by increasing Type I errors. A means to address this could be by cross-validation. This is where the cohort is divided randomly into two groups. One sub-group is then used to determine the optimum cut-point, which is then applied to the second sub-group. The disadvantage to this approach is in reducing the statistical power by halving the size of the study sample.

Histological grade and lymphovascular invasion failed to be identified as independent predictors of disease free survival in this study, despite their role as prognostic factors.
being well-established in the literature to the point of being considered high-risk features of stage II CRC when selecting patients for adjuvant chemotherapy\(^{100, 101, 332}\). Furthermore, the TNM stage was identified as an independent predictor of disease free and overall survival when the entire cohort was examined, but was not identified as a predictor of either in the sub-group of patients with available CEA data. This brings into question the validity of the second part of this study given that TNM stage is considered the strongest and most widely studied prognostic indicator in this setting. However, it should be noted that, given the extent of missing CEA data, the cohort size was significantly reduced in size with only four out of ten patients with stage IV being included. Therefore, it is more likely that the four TNM stages were not adequately represented in the second part of this study, than pre-operative CEA being a stronger predictor than TNM stage.

Another limitation is that the observed recurrence rates may have been influenced by adherence to the post-operative surveillance protocol. Therefore, had the outcomes reported in this study been discordant with other National data, evaluation of the compliance with post-operative surveillance would have been warranted. On the other hand, some studies have failed to demonstrate an improved rate of recurrence detection with increasing surveillance intensity\(^ {352, 353}\). This includes Snyder et al’s retrospective study of 8529 patients across 1175 hospitals\(^ {353}\). The hospitals were determined to be either high- or low-intensity surveillance facilities based on the number of surveillance scans or CEA tests carried out. No significant difference in time-to-detection of recurrence was found nor the proportion of recurrences resected at high- versus low-intensity facilities\(^ {353}\).
2.6 Conclusion

This study found the overall recurrence rates at the host institution to be comparable to International standards, with rectal cancer recurrence rates that were found to be lower.

Recurrences were most commonly observed at distant sites and within two years of surgery. It is possible that these early, distant recurrences indicate the presence of occult metastasis at the time of surgery, in a subset of patients. In turn, circulating biomarkers may provide the sensitivity required to detect such patients and pre-operative carcinoembryonic antigen (CEA) was evaluated as part of this study, given it is a readily available test in clinical practice. We found that a raised pre-treatment CEA was predictive of a poorer overall survival and disease-free survival independent of stage. This corroborates findings within the wider literature and CEA remains the single most studied circulating biomarker in the field of colorectal cancer prognosis. Unlike pathological staging criteria such as lymph node involvement, circulating biomarkers may provide prognostic information that is available to the patient and clinician at the outset. This in turn may help to inform and guide major treatment decisions at an earlier time-point.

As outlined in the first chapter, a growing appreciation of the link between systemic inflammation and outcomes in colorectal cancer is being made through both experimental data and clinical studies. Cytokines, through a number of systemic inflammatory pathways, may act on tumour and host cells to promote tumour growth and dissemination. In the next chapter, a systematic review of the literature will be carried out to evaluate the prognostic value of circulating inflammatory cytokines.
Chapter Three
Prognostic value of multiple cytokine analysis in colorectal cancer: Systematic Review
3.1 Introduction

In Chapter Two, CEA was shown to provide prognostic information in terms of disease free and overall survival. While CEA remains the most widely studied circulating prognostic biomarker in colorectal cancer, the mechanism for this association remains poorly understood. In contrast to this, experimental data suggests circulating cytokines act through a range of pathways involved in tumour progression (Chapter One). This has led to a great deal of interest in studying the prognostic value of circulating cytokines in colorectal cancer. In 2014, a systematic review was performed by Liu et al of prognostic studies of circulating biomarkers in colorectal cancer. They included 49 studies studying 44 prognostic markers but found that only 21 markers were studied in two or more studies. Although their review was not exclusive to inflammatory cytokines, but included tumour markers as well, their findings demonstrate the lack of heterogeneity in the cytokines tested in prognostic biomarker studies.

Despite this, a number of studies have found particular cytokines to show promise as potential prognostic markers, including interleukin-6 (IL-6)\(^{298, 302, 355-357}\), TNF alpha (TNF\(\alpha\))\(^{302, 358, 359}\) and interleukin 1\(\beta\) (IL-1\(\beta\))\(^{302, 360}\). Many of these studies have evaluated individual biomarkers and have found them to weakly predict prognosis. However, experimental data also shows individual cytokines to participate in numerous and sometimes opposing pathways along disease progression\(^{193, 211, 361}\). Therefore, it is possible that characterising inflammatory state by combining multiple cytokines may be a more robust approach than measuring a single inflammatory marker.

Aims

The aims of this chapter are:
To perform a systematic review of the literature evaluating the prognostic value of multiple cytokine analysis in colorectal cancer

To determine whether a composite inflammatory score derived from multiple peripheral cytokine markers provides prognostic value in colorectal cancer

3.2 Methods

Search strategy

The methodology of this systematic review followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement guidelines. Searches of the MEDLINE, EMBASE and Scopus databases were performed on 30th August 2017 using a search strategy that included the terms ‘colon, rectal, colorectal cancer’, and ‘cytokine, cytokines’ and ‘outcome, prognosis, survival, mortality, death, recurrence’. The results were limited to English language, human studies and articles published since the year 2000; studies prior to this were not included due to outdated clinical practices. The corresponding author was contacted in instances where only the abstract was available and the study was subsequently excluded if the available data was incomplete or could not be provided.

Eligibility criteria

Studies examining the association between baseline, peripherally circulating cytokine levels and prognosis in patients with colorectal adenocarcinoma were eligible. Studies were excluded if two or fewer cytokines were evaluated, or the primary outcome measured was the response to chemotherapy.
**Method of study review**

Data was collected in duplicate by two independent reviewers and a third reviewer consulted on areas of differing opinion. Quality assessment of eligible studies was performed by assessing the risk of bias in the six domains described by Hayden et al\(^{364}\).

These were, ‘study participation’, ‘study attrition’, ‘prognostic factor measurement’, ‘outcome measurement’, ‘study confounding’, ‘statistical analysis and reporting’. Two reviewers assessed each study independently and each domain was given a score of 0 for high risk, 1 for moderate risk and 2 for low risk of bias. Instances of differing opinion were resolved through discussion with a third reviewer available to adjudicate if necessary.

### 3.3 Results

**Description of Studies.**

Seven studies were included in this review\(^ {293, 302, 306, 365-368}\) after screening 570 records, the number of records screened out by stage are given with the reasons in the flowchart (Figure 20).

Overall, the quality of the studies was poor to moderate with QUIPS scores ranging from one to six out of twelve (Table 15). Of the six domains assessed, the studies performed well in prognostic factor measurement with a combined score of ten out of fourteen across the seven studies. The authors often reported standardised methods of blood sampling and detailed the assay methods performed. In contrast the studies performed poorly in the ‘study participation’ domain, with a total score of two. The main reasons for this were small sample sizes, retrospective study design and studies that were limited to the study of stage IV patients.
Figure 20. Flowchart demonstrating study selection for inclusion in systematic review. Modified from the PRISMA statement. *Authors of abstracts were contacted.
Table 15. Quality in Prognostic Studies (QUIPS) Scores for studies included in systematic review.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study participation</th>
<th>Study attrition</th>
<th>Prognostic factor measurement</th>
<th>Outcome measurement</th>
<th>Study confounding</th>
<th>Statistical analysis and reporting</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaminska\textsuperscript{368}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Shimazaki\textsuperscript{293}</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chen, 2015\textsuperscript{a365}</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Chen, 2015\textsuperscript{b371}</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Vayrynen\textsuperscript{100}</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Chang\textsuperscript{302}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Di Caro\textsuperscript{367}</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

0: High risk, 1: Moderate risk, 2: Low risk of bias.
Five of the seven studies included patients of all stages (Table 16) \(^{293,302,306,367,368}\). Two of these included all patients attending hospital with a histological new diagnosis of colorectal adenocarcinoma\(^{302,368}\) and three studies included only those undergoing surgery\(^{293,306,367}\). The remaining two studies included stage IV patients only\(^{365,366}\) although a clear rationale for looking exclusively at this sub-group was not given, citing evidence linking circulating cytokines to prognosis in colorectal cancer across patients of all stages\(^{207,298,370}\).

Two studies were prospective in design\(^{306,367}\), two retrospective\(^{302,366}\) and the study design was not stated in three studies\(^{293,365,368}\). The sample sizes were relatively modest ranging from 46 to 205 participants and all seven studies were conducted at separate, single institutions. None of the investigators reported an attempt to perform power calculations to arrive at their intended sample size. Five of the seven studies had a mean or median follow up duration of approximately five years.

The number and selection of cytokines measured varied across the studies and whilst IL-6 was evaluated in all seven and IL-8 evaluated in six studies\(^{293,306,365-368}\) based on existing literature, lesser known markers were included in some of the, particularly larger, panels tested\(^{365,366}\). The Chen et al (2015a\(^{365}\) and 2015b\(^{366}\)) studies utilised commercially available panels including 39 and 51 cytokines, providing justification for only a fraction of the markers tested in the form of existing scientific and clinical evidence. All seven studies measured cytokine levels in pre-treatment samples, with no comparisons to post-operative or follow-up specimens.
Table 16. Summary of studies included in systematic review including individual cytokines and outcomes measured

<table>
<thead>
<tr>
<th>Author</th>
<th>Population, Year</th>
<th>n</th>
<th>Stage</th>
<th>No. of cytokines</th>
<th>Cytokines measured</th>
<th>Follow Up (months)</th>
<th>Outcome</th>
<th>QUIPS Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaminska</td>
<td>Poland, 2005</td>
<td>157</td>
<td>All stages</td>
<td>14</td>
<td>IL-1b, IL-6, TNFα, IL-8, sIL-1Ra, sIL-6R, sTNF R1, sTNF RII, IL-1α, IL-10, G-CSF, M-CSF, GM-CSF</td>
<td>Median 60</td>
<td>OS</td>
<td>3</td>
</tr>
<tr>
<td>Shimazaki</td>
<td>Japan, 2013</td>
<td>46</td>
<td>All stages</td>
<td>6</td>
<td>IL-1, IL-6, IL-8, TNFα, G-CSF, M-CSF</td>
<td>Mean 70.3</td>
<td>OS</td>
<td>1</td>
</tr>
<tr>
<td>Chen</td>
<td>China, 2015a</td>
<td>176</td>
<td>Stage 4 only</td>
<td>39</td>
<td>IL-1A, IL-1B, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-13, IL-15, IL-17, IL-12P40, IL-12P70, EGF, Eotaxin, G-CSF, GM-CSF, IFNA2, IFN?, IP-10, MCP-1, MCP-3, MIP-1A, MIP-1B, TNFA, TNFB, VEGF, FGF-2, TGF-α, FLT-3L, GRO, MDC, SCDF40L, SIL-2RA</td>
<td>Median 19.6</td>
<td>OS</td>
<td>6</td>
</tr>
<tr>
<td>Chen</td>
<td>USA, 2015b</td>
<td>205</td>
<td>Stage 4 only</td>
<td>51*</td>
<td>IL-6, IL-8, IL-2Ra, HGF, M-CSF, VEGF-A, IL-12, MIP-1B, GROα, IL-18, MIF, SCGF-B, TRAIL, Amphiregulin, EGF, Epiregulin, HB-EGF, Tenascin, TGF-α, ICAM-1</td>
<td>Mean/median not reported</td>
<td>OS</td>
<td>5</td>
</tr>
<tr>
<td>Vayrynen</td>
<td>Finland, 2016</td>
<td>147</td>
<td>All stages</td>
<td>13</td>
<td>IL-1α, IL-4, IL-6, IL-7, IL-8, IL-9, IL-12, IFN?, CXCL10, CCL2, CCL4, CCL11, PDGFB-B</td>
<td>Median 59.2</td>
<td>OS, DFS, CSS</td>
<td>6</td>
</tr>
<tr>
<td>Chang</td>
<td>Taiwan, 2016</td>
<td>164</td>
<td>All stages</td>
<td>3</td>
<td>IL-1b, IL-6, TNFα</td>
<td>Mean 64.9</td>
<td>DFS</td>
<td>3</td>
</tr>
<tr>
<td>Di Caro</td>
<td>Italy, 2016</td>
<td>69</td>
<td>All stages</td>
<td>8</td>
<td>IL-1, IL-6, IL-10, TNFα, CCL2, IL-8**, VEGF, PTX</td>
<td>Mean 56.3</td>
<td>DFS</td>
<td>6</td>
</tr>
</tbody>
</table>

The primary end-point measured was overall survival in four studies, and in the remaining three studies, was based on the incidence of disease recurrence. The study by Chang et al referred to this as the progression-free survival, which was defined as ‘no imaging or pathological evidence of disease progression’. The study included patients of all stages and the treatments undertaken by participants were not stated in the report. Väyrynen et al measured the Disease Free Survival (DFS) but did not provide a definition of this outcome in the manuscript and Di Caro et al measured DFS defining their events as, ‘any event of local tumour recurrence or any metachronous distant-organ metastases’. For simplicity, the results of these three studies have been grouped together as ‘disease free survival’ in Table 17.

Di Caro et al utilised computed tomography, ultrasound and chest radiographs according to a common protocol, and was the only study to report a standard approach to the follow up of their cohort.
Table 17. Summary of results of studies included in systematic review.

<table>
<thead>
<tr>
<th>Study</th>
<th>Prognostic marker</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaminska 2005&lt;sup&gt;368&lt;/sup&gt;</td>
<td>sTNF RI</td>
<td>RR 3.10 (Relative Risk)</td>
<td>0.009</td>
</tr>
<tr>
<td>Shimazaki 2013&lt;sup&gt;293&lt;/sup&gt;</td>
<td>IL-6</td>
<td>HR 4.1 (1.20-13.98)</td>
<td>0.024</td>
</tr>
<tr>
<td>Chen 2015a&lt;sup&gt;*&lt;/sup&gt;&lt;sup&gt;365&lt;/sup&gt;</td>
<td>Flt-3L</td>
<td>HR 2.19 (1.29-3.71)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>MDC</td>
<td>HR 1.89 (1.29-2.77)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>IL-2</td>
<td>HR 1.71 (1.14-2.56)</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>IL-8</td>
<td>HR 2.06 (1.28-3.32)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>HR 1.69 (1.11-2.59)</td>
<td>0.015</td>
</tr>
<tr>
<td>Chen 2015b&lt;sup&gt;*&lt;/sup&gt;&lt;sup&gt;371&lt;/sup&gt;</td>
<td>†CS</td>
<td>HR 2.29 (1.51-3.48)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NLR-CS</td>
<td>HR 2.09 (1.59-2.76)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vӓyrynen 2016&lt;sup&gt;306&lt;/sup&gt;</td>
<td>IFNy</td>
<td>HR 0.47 (0.24-0.90)</td>
<td>0.022</td>
</tr>
</tbody>
</table>

**Independent Predictors of Disease Free Survival**

<table>
<thead>
<tr>
<th>Study</th>
<th>Prognostic marker</th>
<th>Hazard Ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chang 2016&lt;sup&gt;302&lt;/sup&gt;</td>
<td>††CS</td>
<td>HR 9.20 (1.21-69.70)</td>
<td>0.036</td>
</tr>
<tr>
<td>Di Caro 2016&lt;sup&gt;367&lt;/sup&gt;</td>
<td>IL-8</td>
<td>HR Not available</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>PTX3</td>
<td>HR 9.64 (2.24-41.42)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>HR Not available</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>††† CS</td>
<td>HR 16.21 (3.56-73.84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vӓyrynen 2016&lt;sup&gt;306&lt;/sup&gt;</td>
<td>CCL4</td>
<td>HR 0.38 (0.15-0.92)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

No cytokines identified as independent predictors of Cancer Specific Survival.


*Stage IV only
† Cytokine score based on IL-6,IL-8,IL-2Ra,IL-18,HGF,M-CSF,VEGF-A,MIP-1b,GROa,MIF,SCGF-b,TRAIL,Amphiregulin,EGF,Epiregulin,HB-EGF, Tenascin,TGF-a,ICAM-1.
†† Cytokine score based on IL-6,IL-1β and TNFα. In patients with CRP<5 mg/L
††† Cytokine score based on IL-8,VEGF & Pentraxin-3.
Individual cytokines and Overall Survival.

Five studies measured overall survival as the primary outcome\(^\text{293, 306, 365, 366, 368}\). On univariate analysis, Chen et al (2015a) found 17/39 (43.6\%)\(^\text{365}\), Kaminska 6/14 (42.8\%)\(^\text{368}\), Shimazaki 1/6 (16.7\%)\(^\text{293}\) and Vӓyrynen found none of 13 cytokines\(^\text{306}\) to be associated with OS whilst Chen et al (2015b) did not report the results of univariate analysis. On multivariate analysis, Chen et al (2015a) found 5/39 (12.8\%)\(^\text{365}\), Chen et al (2015b) none of 51\(^\text{366}\), Kaminska et al none of 14\(^\text{368}\), Shimazaki et al 1/6 (16.7\%)\(^\text{293}\) and Vӓyrynen found 1/13 (7.7\%)\(^\text{306}\) individual cytokines measured to independently predict OS. All five studies included IL-6 in their panel and only one of these identified this cytokine as an independent predictor\(^\text{293}\). All five studies included IL-8 and none of them identified it as a predictor (Table 17).

One study found that raised levels of interferon gamma (IFNγ) were predictive of a favourable overall survival\(^\text{306}\). IFNγ was also included in the panel of one other study - which was limited to stage IV patients - and did not show a significant association with OS on univariate analysis and therefore was not included in multivariate analysis\(^\text{365}\). One other marker, CCL4, was identified across the seven studies to predict improved survival with increased levels\(^\text{306}\).

Individual cytokines and Disease Free Survival.

Three studies measured disease free survival. Di Caro et al found 3/6 (50\%)\(^\text{367}\), Chang et al 2/3 (66.6\%)\(^\text{302}\) and Vӓyrynen found 1/13 (7.7\%)\(^\text{306}\) cytokines to be associated with DFS on univariate analysis. On multivariate analysis, Di Caro et al found 3/8 (37.5\%),\(^\text{367}\) Chang et al none of three\(^\text{302}\) and Vӓyrynen found 1/13 (7.7\%)\(^\text{306}\) individual cytokines to predict DFS. All three studies included IL-6 in their panel but none went on to identify this cytokine as
an independent prognostic indicator. IL-8 was found to independently predict DFS in one of the two studies that measured this cytokine (Table 17).

**Combined ‘Cytokine Scores’**

Four studies combined multiple cytokine levels into a composite score and varied in the number and selection of cytokines as well as the way in which they were combined.

Di Caro et al incorporated three cytokines that were independent predictors of DFS on multivariate analysis. Using cut-off values identified on ROC curve analysis, a ‘high’ score was given if all three markers were raised, a ‘low’ score for patients where all three markers were low and a ‘medium’ score for the remaining patients. This score improved upon the prognostic value of Pentraxin-3, an acute phase protein related to C reactive protein. The hazard ratios were not available for the remaining two cytokines as all nine events occurred in the patients with raised levels. This meant that a direct comparison between individual cytokines and the composite cytokine score was not possible.

Chang et al measured three cytokines and incorporated all three into the cytokine score despite only two of which showed a significant association with OS on the Chi squared test. Patients were assigned a cytokine score of zero to three based on the number of cytokine values that were above the median. This cytokine score outperformed individual cytokine levels in patients with a CRP of less than 5mg/L although in patients with CRP above 5mg/L, individual cytokines and the composite cytokine score were not independent predictors of DFS. The investigators did not justify a rationale for dividing their cohort into high and low CRP groups but did propose cytokine intensity as an alternative indicator of inflammation in colorectal cancer patients.
Chen et al (2015a) included 17 cytokines that were predictive of overall survival, on univariate analysis, into their cytokine score. The investigators assigned each cytokine a weighted score, based on their hazard ratio on cox proportional regression. The optimum cut-off value for the cytokine score was then identified from Receiver Operator Characteristic (ROC) curve analysis to dichotomise the variable into high and low. The investigators did not evaluate their score against important co-variates such as patient and tumour characteristics in a multivariate model but did report a sensitivity and specificity in predicting OS of 0.833 and 0.737, respectively. This out-performed all individual cytokines in its prognostic accuracy.

Chen et al (2015b) identified 19 cytokines that correlated with neutrophil-lymphocyte ratio and incorporated them into their cytokine score. Each patient was given a score based on the sum of the Z scores of the selected cytokines. The Z score was defined as the difference of the average and individual cytokine level divided by standard deviation of log2-transformed value. The cohort was then divided into high or low cytokine score groups using the median score as a cut-off which predicted OS. Individual cytokines were not entered into a cox proportional regression analysis and were therefore not compared directly against the composite cytokine score.

All four studies that included a cytokine score concluded that the scores were prognostic. However, only Chen et al (2015b) made a direct comparison between their composite score and individual cytokines, demonstrating an enhanced prognostic ability.
3.4 Discussion

This systematic review found that only seven studies evaluated multiple cytokines after 570 records were screened. A high degree of heterogeneity was found between the studies with respect to the patient groups evaluated and outcomes measured. They were also limited by small sample sizes and included those taking an opportunistic look at retrospective cohorts, contributing to poor to moderate quality scores. The use of large, pre-manufactured cytokine panels was sometimes chosen over a targeted investigation of smaller panels of cytokines with known prognostic value and the results of individual cytokine analysis yielded inconsistent findings. In contrast to this, a sub-set of studies combined multiple cytokines to produce a composite cytokine score and these were more consistently found to be predictive, although different methods were used to produce these scores. Furthermore, no reports were found to evaluate different methods of producing a composite score.

The link between inflammation and cancer is considered such that inflammation has been named as one of the hallmarks of cancer\(^{373}\). This is demonstrated by the up to twenty-fold increase in the lifetime risk of CRC in patients with inflammatory bowel disease, and whilst colitis-associated cancer (CAC) accounts for only 2-3% of CRCs, inflammation also plays an important role in sporadic cancers\(^{374, 375}\). On the cellular level, inflammation within the tumour microenvironment facilitates malignant cell survival, growth and progression by the actions of inflammatory cytokines acting through a variety of pathways including the IL-6/JAK/STAT pathway, which is considered key\(^{193, 376}\). Systemic processes are also thought to play an important role both in recruiting immune cells from the bone marrow and spleen and in facilitating the colonisation of distant organs by metastatic deposits\(^{232}\).
Given the emerging role of systemic inflammation in colorectal cancer, it is clinically relevant to evaluate the prognostic value of circulating inflammatory cytokines.

The ability to predict prognosis is of value to clinicians largely by informing treatment decisions. More aggressive treatment strategies, namely the use of adjuvant chemotherapy following surgical resection may reduce the likelihood of recurrence but results in exposure to potentially harmful side effects and must be administered selectively to those patients considered high-risk. At present, the pathological TNM stage provides the most powerful means of risk-stratifying patients but uncertainty remains, particularly in patients with stage II disease who have a 5-year recurrence rate of approximately 20% \(^{377, 378}\). Two of the included studies were limited to stage IV patients only and a clear rationale for this was not included in the reports \(^{365, 366}\). The results of these studies do not therefore contribute to answer the question of whether multiple cytokines measurement can provide prognostic information that may be used to inform treatment strategy. The two studies limited to stage IV patients also contained variation in the treatment regimes received by participants. In the Chen et al’s (2015b) study \(^{366}\), 39 patients received fluorouracil, irinotecan and bevacizumab as part of a phase II clinical trial and the treatment undertaken by the remaining 166 patients was not described \(^{379}\). The patients included in the other study by Chen et al (2015a) all underwent systemic chemotherapy as their primary treatment and 19 patients underwent partial liver resection, suggesting the cohort included patients undergoing a palliative as well as radical treatment strategy \(^{365}\). This heterogeneity in study populations diminishes the generalisability of their findings.
The sample sizes were relatively small, ranging from 46 to 205. Furthermore, power calculations were not reported in any of the studies of which only two were prospective in design. The likelihood of type II error may also have been increased by introducing an excessive number of co-variates into the multivariate analyses; a widely accepted rule of thumb being a minimum of ten events being required per variable. Recently, calls have been made to relax this rule in certain situations although this is mainly applicable to tests of sensitivity rather than prognostic studies. In addition to the number of variables included, the variables themselves are important to consider.

Given that the TNM classification system is widely used to inform treatment strategies, this provides an ideal benchmark to compare cytokine profiles against, yet three studies did not include TNM stage as a co-variate in multi-variate analysis. In addition to this, cytokine levels have been shown to vary with stage in clinical studies and for these reasons, all prognostic studies of cytokine levels should control for this variable.

Although overall survival is considered the gold standard outcome in prognostic studies, disease free survival is also an important outcome to measure and is of clinical interest as it reflects disease recurrence. Three of the studies included in this review reported disease free survival as the primary outcome although it is important to acknowledge that the definition of this outcome varied between them. Additionally, a systematic review by Punt et al included 52 studies of adjuvant treatment in colon cancer and found wide variation in the definition of the endpoints used and the starting point for measuring time to events. This variation in the end-point definitions used must be taken into account when interpreting the DFS results.
All seven of the included studies cited evidence for an overlapping selection of cytokines, frequently including IL-6, IL-8, IL-1β and TNFα, linking systemic inflammation and outcome in colorectal cancer to justify the rationale of their study and yet, in contrast, varied widely in the number and selection of cytokines included in the panels they went on to study. Studies including the lesser known cytokines in their panels were often those implementing multiplex cytokine assays using commercially available, pre-manufactured cytokine panels. This approach resulted in a generally low-yield in identifying novel biomarkers, one such example of which is Fms-related tyrosine kinase 3 ligand (Flt-3L), shown to independently predict overall survival by Chen et al (2015a). Flt-3L is a growth and differentiation factor generally associated with haematopoiesis and has been found to predict poor prognosis in acute myeloid leukaemia although its prognostic value in colorectal cancer has not otherwise been reported.

Two cytokines with a more established prognostic role in colorectal cancer are IL-6 and IL-8. Whilst these cytokines were included in the majority of panels tested, they were only identified as independent predictors in two of the studies. A possible explanation for this is that the predictive power of individual cytokines negate each other due to the high level of co-variance between them. This is especially true of studies including multiple cytokine levels each as co-variates in multivariate analysis.

The four studies that combined multiple cytokine values into a single score all utilised different methods. The question of which cytokines to include is the first consideration and Di Caro et al’s approach of selecting those cytokines that were predictive of the endpoint independent of stage seems logical. In contrast, Chang et al’s inclusion of a cytokine that had no individual association with outcome would be unlikely to strengthen
the predictive power of their combined score\textsuperscript{302}. The cut-off values used for individual cytokines were either taken as the median, having the advantage of dividing the cohort into equal groups, or were identified through ROC curve analysis. The latter approach is preferable where the data is skewed and this has often been shown to be the case with inflammatory cytokine levels in colorectal cancer populations\textsuperscript{382,383}. Individual cytokines were weighted according to effect size in the scores used by Chen et al (2015a) and Chen et al (2015b)\textsuperscript{365,366}. This approach acknowledges the finding that some cytokines are more powerful predictors of outcome than others, a theme echoed in the results of each of the studies included in this review as well as many within the wider literature. The finding that two cytokines, IFNγ and CCL4, were found to predict improved rather than worsened survival, in contrast to the remaining prognostic cytokines, only emphasises the need to consider the differential relationships between cytokine value and outcome when formulating a combined score. While the data suggests a multi-marker approach may be useful, no real guidance is provided on how to combine them.

Limitations

The main limitation of this study pertains to the exclusion of studies measuring fewer than three cytokines. By doing so, the validity of comments drawn on the prognostic value of individual cytokines are impeded by a significant selection bias. The number and breadth of studies into the prognostic value of single cytokine markers, apparent from an initial search of the existing literature as well as the review carried out by Liu et al\textsuperscript{354}, suggested that encompassing them all into a systematic study would be unlikely to be of great value. This is especially true when weighed up against the practicality of performing such a
However, the study could have been improved by including studies measuring two cytokines, some of which may have utilised a combined score.

It is noteworthy that, while this study may have been useful in drawing general conclusions about the current literature, the data fell far short of allowing any pooled analysis to be performed. Ideally to do this, the raw data from a number of studies demonstrating low heterogeneity between cohorts would need to be pooled into a single Cox proportional hazard model including potentially confounding variables. Additionally, the combined cytokine score would have to be composed of the same cytokines and by the same method in each of the included studies. The extent and quality of the available reported data did not allow such an analysis but could be performed in the future if data availability allows.

3.5 Conclusion

This review demonstrates a paucity and heterogeneity of studies examining the prognostic value of combining circulating cytokine markers in CRC. There is therefore a need for well-designed prospective studies evaluating a panel of cytokines that is reasonable in number and justified in their inclusion.

From this review, prognostic studies evaluating panels of multiple cytokines are relatively ineffective in identifying novel biomarkers and inconsistent in validating more established predictors individually. Despite these forthcomings, there may be some promise in combining multiple cytokines to produce an enhanced, predictive score.
Chapter Four

Circulating cytokine profiles

by disease status
4.1 Introduction

As outlined in Chapter One, systemic inflammation has been implicated in a number of pro-metastatic cellular pathways\textsuperscript{193, 232}. These include systemic-signalling between the primary tumour and pre-metastatic or metastatic sites that enhance invasion, intravasation and metastatic colonisation\textsuperscript{232}. In the previous chapter, a systematic review was performed evaluating the prognostic value of multiple cytokine analysis. Here, the combination of multiple markers tended to provide additional prognostic information to disease stage alone. However, one of the major design flaws in the included studies was the inclusion of large panels of pre-manufactured panels of cytokines, some of which had little or no previous evidence linking them to colorectal cancer progression. Although the systematic review was not directly useful in selecting the cytokines of interest for the present study, it informed our approach of testing a small panel of deliberately selected markers. Four cytokines were selected on the basis of having an established link with CRC progression, from experimental data.

Interleukin-6 is a pleotropic inflammatory marker and has demonstrated roles in tumour progression through cancer cell survival\textsuperscript{386}, promoting angiogenesis\textsuperscript{213} and accumulation of MDSCs within tumours\textsuperscript{387}. Interleukin-8 is a chemokine involved in neutrophil mobilisation to tumours which in turn promotes tumour progression\textsuperscript{388}. Studies in CRC cell line models have demonstrated IL-8 to promote tumour growth and metastasis\textsuperscript{207}. TNF-\alpha promotes MDSC survival\textsuperscript{389} and triggers proliferation of intestinal epithelial cells and CRC cell lines by acting on the TNFR2 receptor\textsuperscript{390}. Interleukin-1\beta is expressed by tumour associated macrophages\textsuperscript{197} and neutrophils\textsuperscript{196} and may act directly on tumour cells to stimulate their proliferation\textsuperscript{197}. 

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For a marker to have prognostic value, we would expect a clear difference in the levels of that marker between healthy controls and any colorectal cancer and to show a difference between those with and without metastatic disease. CEA and CRP are widely studied circulating prognostic biomarkers in CRC and were therefore used for comparison against our chosen cytokine panel 298, 391-393.

Aims

The aims of this study are:

❖ To compare baseline levels of plasma IL-6, IL-1β, TNFα and IL-8 between the three groups: healthy control, stage II and stage IV CRC.
❖ To compare cytokine profiles with C-reactive protein (CRP) and carcinoembryonic antigen (CEA)
❖ To utilise a combined score that optimises sensitivity and specificity to differentiate between healthy controls, localised and metastatic CRC.

4.2 Methods

Patient selection.

Patients were recruited prospectively into this study from Wellington Hospital, New Zealand over a sixteen-month period between October 2016 and February 2018. Disease stage was assigned according to the 8th edition of American Joint Committee on Cancer guidance 1. Clinical data were de-identified and stored securely on the University server using the software REDCap v8.6.4 (Vanderbilt University, Tennessee USA) 394.
Healthy controls

Healthy control participants were enrolled from elective endoscopy lists immediately prior to undergoing a colonoscopy that was requested on the suspicion of lower gastrointestinal malignancy. Patients were excluded if any evidence of inflammation or malignancy was detected and were also excluded if they had significant comorbidities including chronic inflammatory conditions or synchronous malignancy.

Stage II Patients

Patients were recruited into the stage II group on the basis of radiological staging. Rectal cancer patients underwent pre-operative MRI pelvis and CT chest abdomen and pelvis, and colon cancer patients underwent pre-operative CT chest, abdomen and pelvis alone. Imaging was reviewed by two Consultant Radiologists. For colon cancers, staging was confirmed postoperatively by pathology.

Stage IV Patients

Consecutive patients presenting with metastatic colorectal cancer at diagnosis were recruited into the stage IV group.
Exclusion Criteria

Exclusion criteria were:

- age below 18 years
- emergency presentation
- chronic inflammatory conditions including inflammatory bowel disease
- other synchronous primary malignancy
- colorectal cancer other than adenocarcinoma
- use of immunosuppressive therapy including oral corticosteroids.

Ethics

All patients recruited into this study gave their written informed consent and ethics approval was granted by the Health and Disability Ethics Committee (HDEC ref: 18/CEN/138).

Plasma sample collection and storage.

After written consent was obtained, pre-treatment venous blood samples were withdrawn from patients in the outpatient setting. Blood withdrawal was performed in the morning where possible in a non-fasting state and within four weeks of initial treatment. In instances when a delay to initial treatment occurred, a repeat sample was taken. In patients undergoing neo-adjuvant therapy, blood samples were withdrawn prior to any treatment commenced. Twenty millilitre venous blood specimens were withdrawn using a 23G BD™ vacutainer blood collection set into sodium citrate vacutainer tubes after discarding the first 5 mL. Venepuncture was preferentially performed from the antecubital fossa and samples were centrifuged within 30 minutes of withdrawal at 2000 xg for ten
minutes. Haemolysed samples were discarded. Plasma was stored at -80 degrees Celsius for a maximum of twenty-four months. No plasma samples underwent more than two freeze-thaw cycles prior to analysis.

**Cytokine assays.**

All assays were performed within twenty-four months of sample collection and in two or fewer freeze-thaw cycles. Levels of IL-1β, IL-6, IL-8 and TNFα were quantified from the plasma using commercially available sandwich ELISA kits specific for each cytokine (Invitrogen, ThermoFisher Scientific) as per the manufacturers instructions. Absorbances were measured using the “Multiskan GO” (Thermo Scientific) specialised spectrophotometer. The detection limits were IL-6: 2-200 pg/mL, IL-1β: 2-150 pg/mL, IL-8: 2-250 pg/mL and TNFα: 4-500 pg/mL.

Blood obtained at the same venepuncture were also sent to the clinical diagnostics laboratory at Wellington Hospital where the following were measured, CEA (Roche Cobas e601, reference range <3.5 µg/L) and CRP (Roche Cobas 8000, reference range <6 mg/L).

**Statistical analysis**

Statistical analysis was performed as described in Chapter Two (2.2). In addition to this, cytokine markers were compared using the area under the curve (AUC) on receiver operator curve (ROC) analysis. ROC curves were plotted for each marker’s ability to discriminate between controls versus stage II, stage II versus stage IV and controls versus stage IV.
**IL8-CEA combined score**

IL-8 and CEA values were log-transformed and expressed as a fraction of the highest value. This gave a score of zero to one for IL-8 and for CEA, which were then combined to produce a sum of zero to two.

### 4.3 Results

A total of sixty patients were included in the study across three groups: control (n=19), stage II (n=22) and stage IV (n=19). The mean age was 65.9 years (range 29-85) with 33 (55.0%) males and 27 (45.0%) females. By ethnicity, 53 (88.3%) were European, 4 (6.7%) Maori and 3 (5.0%) other. The median body mass index (BMI) was 28.2 kg/m$^2$ (IQR, 25.0-31.7). There was a lower proportion of patients with ischaemic heart disease in the stage IV group compared with controls (p=0.05) and this was found to be the only patient characteristic to vary significantly between the three groups (Table 18).
Table 18. Demographics of patients included in prospectively recruited cohort, by disease status (Control, Stage II and Stage IV).

<table>
<thead>
<tr>
<th></th>
<th>Control n= 19 (%)</th>
<th>Stage II n= 22 (%)</th>
<th>Stage IV n= 19 (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean +/- SD</td>
<td>68.0 ± 12.0</td>
<td>66 ± 12.0</td>
<td>63.0 ± 12.0</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7 (36.8)</td>
<td>15 (68.2)</td>
<td>11 (57.9)</td>
<td>0.13</td>
</tr>
<tr>
<td>Female</td>
<td>12 (63.2)</td>
<td>7 (31.8)</td>
<td>8 (42.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
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</tr>
<tr>
<td>European</td>
<td>19 (100)</td>
<td>19 (86.4)</td>
<td>15 (78.9)</td>
<td>0.12</td>
</tr>
<tr>
<td>Non-European</td>
<td>0 (0)</td>
<td>3 (13.6)</td>
<td>4 (21.1)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>28.2 (24.8-32.4)</td>
<td>29.6 (26.9-33.1)</td>
<td>27.0 (23.7-29.8)</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Current smoker</strong></td>
<td>0 (0)</td>
<td>2 (9.1)</td>
<td>4 (21.1)</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>IHD</strong></td>
<td>4 (21.1)</td>
<td>1 (4.5)</td>
<td>0 (0)</td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td><strong>DM2</strong></td>
<td>3 (15.8)</td>
<td>3 (13.6)</td>
<td>3 (15.8)</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Aspirin</strong></td>
<td>6 (31.6)</td>
<td>5 (22.7)</td>
<td>2 (10.5)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

BMI: body mass index, IHD: ischaemic heart disease, DM2: type two diabetes mellitus, SD: Standard deviation, IQR: Interquartile range.
Patient groups

Control patients.

Nine of the 19 control patients had polyps on colonoscopy. Histology revealed tubulovillous adenoma (n=4), tubular adenoma (n=5), sessile serrated adenoma (n=3) and hyperplastic polyps (n=2). Three patients had more than one polyp-type found.

Stage II patients.

Nine out of 22 patients in the stage II group had rectal cancer. All were recruited after initial staging. Seven patients had neo-adjuvant therapy (six had short course radiotherapy and one had long course chemo-radiation), with four then re-classified as stage I based on post-operative histology. One patient was found to have stage III based on post-operative histology.

Stage IV patients.

Thirteen patients had liver metastases of which four had a solitary lesion, two had multiple lesions confined to one lobe and seven patients had multiple lesions involving both lobes of the liver. Three patients had lung metastases, six patients had distant lymph node involvement and two patients had peritoneal involvement. Five patients had more than one organ involved.

Cytokines and patient characteristics

IL-6 levels were detected in all patients and IL-8 in 59/60 (98.3%) patients whereas TNFα was only detectable in 8/60 (13.3%) of patients and IL-1β was detectable in no patients. Therefore, TNFα and IL-1β were excluded from further analyses.
When the Shapiro-Wilk test of normality was applied, none of the distributions of IL-6, IL-8, CEA or CRP were found to be normal (p<0.001). Therefore, non-parametric statistical testing was used to compare each marker by patient characteristic (Table 19). CEA values were found to be higher in subjects of non-European ethnicity (median 19.90 µg/L) compared with patients of European ethnicity (median 3.00 µg/L) and this was found to be statistically significant (p=0.05). CEA values also appeared higher in current smokers, patients with high BMI and patients taking regular Aspirin, although these differences were not significant. There were no other significant associations between biomarker levels and patient characteristics.
Table 19. IL-6, IL-8, CEA and CRP values by patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>IL-6, pg/mL</th>
<th>Median (IQR)</th>
<th>P value</th>
<th>IL-8, pg/mL</th>
<th>Median (IQR)</th>
<th>P value</th>
<th>CEA, μg/L</th>
<th>Median (IQR)</th>
<th>P value</th>
<th>CRP, mg/L</th>
<th>Median (IQR)</th>
<th>P value</th>
</tr>
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<td>Age, years</td>
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<tr>
<td>Over 65</td>
<td>2.46 (2.01-4.70)</td>
<td>0.17</td>
<td></td>
<td>5.61 (4.09-9.33)</td>
<td>0.32</td>
<td></td>
<td>3.10 (1.90-8.30)</td>
<td>0.64</td>
<td>4.00 (3.00-8.00)</td>
<td>0.14</td>
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<tr>
<td>65 or under</td>
<td>3.46 (2.14-7.69)</td>
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<td></td>
<td>7.72 (4.59-15.01)</td>
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<td></td>
<td>3.10 (1.90-20.50)</td>
<td></td>
<td>7.00 (3.00-17.00)</td>
<td></td>
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<tr>
<td>Male</td>
<td>2.82 (1.41-6.64)</td>
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<td>5.33 (3.62-9.73)</td>
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<td>3.15 (2.00-6.50)</td>
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<tr>
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<td>19.90 (2.40-75.20)</td>
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<tr>
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<tr>
<td>Over 29</td>
<td>2.62 (2.22-5.55)</td>
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<td>6.82 (4.59-10.09)</td>
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<td>Aspirin</td>
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<td>6.40 (4.33-11.40)</td>
<td></td>
<td></td>
<td>3.05 (2.00-19.80)</td>
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<td>6.00 (3.00-12.00)</td>
<td></td>
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</tr>
</tbody>
</table>

BMI: Body mass index; IHD: ischaemic heart disease; DM2: diabetes mellitus type 2
Cytokines and disease stage.

When biomarker levels were compared across the three patient groups, IL-6 (p=0.005), IL-8 (p<0.001), CEA (p<0.001) and CRP (p<0.001) all increased significantly with advancing disease stage (Figure 21 and 22). The increases in IL-6, IL-8, CRP and CEA across stage were most pronounced between stage II and stage IV and the widest range of values were seen in the stage IV group.

Figure 21. Scatter plots of IL-6 (A) and IL-8 (B) values by disease status. *, p<0.05
IL-6 varied significantly between control (median 2.46, IQR 1.85-3.98), stage II (median 2.51 pg/mL, IQR 2.01-3.46 pg/mL) and stage IV (median 6.12 pg/mL, IQR 4.63-10.56 pg/mL), p=0.005. IL-8 also varied significantly between the three groups: control: median 3.95 pg/mL, IQR 3.35-6.51 pg/mL; stage II: median 5.59, IQR 4.33-9.27 pg/mL; stage IV: median 11.40, IQR 8.39-34.05 pg/mL, p<0.001 (Figure 21).

CRP increased across the three groups. Control: median 3.00 mg/L (IQR, 3.00-7.00 mg/L); Stage II: median 4.00 (IQR, 3.00-9.00); stage IV: median 11.00 (IQR, 7.00-54.00), p<0.001. CEA increased across all three groups. Control: median 1.90 µg/L (IQR, 1.20-2.90 µg/L);
stage II: 3.45 µg/L (IQR, 2.00-6.50 µg/L); Stage IV: median 20.50 µg/L (IQR, 4.60-131.20 µg/L), p<0.001 (Figure 2).

Given the wide range of values that were observed in the stage IV group, patients in this group were further subdivided into those with liver involvement (n=13) to those without (n=6). Biomarker levels did not differ significantly between patients with liver involvement and those without: IL-6 (p=0.90), IL-8 (p=1.00), CEA (p=0.52) or CRP (p=0.97) (Figure 23).
Figure 23. Scatter plots of IL-6, (A), IL-8 (B), CRP (C) and CEA (D) among stage IV patients, by presence of liver involvement.
Figure 24. Scatter plots of IL-6 (A), IL-8 (B), CRP (C) and CEA (D) among stage IV patients, by number of organs involved.
The stage IV group was then divided into patients with one organ involvement (n=15) and those with more than one organ (n=4). The biomarkers did not differ significantly according to these sub-groups: IL-6 (p=0.74), IL-8 (p=0.13), CEA (p=0.41) or CRP (p=0.60) (Figure 24).

**ROC curve analysis.**

Receiver operator characteristic curve analyses were performed to evaluate IL-6, IL-8, CRP and CEA as discriminators between patient groups.

CEA was the only individual marker to discriminate between control and stage II disease (p=0.008)(Table 20). All four markers discriminated between stage II and stage IV groups, the most effective being IL-8 (AUC=0.80) and CEA (AUC=0.81) and all four markers were significant discriminators between the control and stage IV groups, again with IL-8 (AUC=0.90) and CEA (AUC=0.92) the most effective.

As the two most effective discriminators between patient groups, IL-8 and CEA were then combined to produce an IL8-CEA score, ranging from possible scores of zero to two. The method by which this combined score was calculated is described in the ‘statistical analysis’ section. The ROC curves (Figure 25) demonstrate the IL8-CEA combined score to be an effective discriminator between each patient group-pairing, out-performing any one marker when compared by area under the curve. The combined score was especially effective in discriminating between the control versus stage IV groups (AUC=0.99).
Table 20. Receiver Operator Characteristic curve analysis of IL-6, IL-8, CRP, CEA and IL8-CEA by disease status comparison.

<table>
<thead>
<tr>
<th></th>
<th>Control v Stage II</th>
<th>Stage II v Stage IV</th>
<th>Control V Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>p</td>
<td>AUC</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.55</td>
<td>0.619</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.68</td>
<td>0.057</td>
<td>0.8</td>
</tr>
<tr>
<td>CRP</td>
<td>0.63</td>
<td>0.163</td>
<td>0.76</td>
</tr>
<tr>
<td>CEA</td>
<td>0.75</td>
<td><strong>0.008</strong></td>
<td>0.81</td>
</tr>
<tr>
<td>IL8-CEA†</td>
<td>0.77</td>
<td><strong>0.003</strong></td>
<td>0.89</td>
</tr>
</tbody>
</table>

AUC: Area under the curve. †IL8-CEA combined score.
Figure 25. Receiver Operator Characteristic curves for Combined IL8-CEA Score by disease status comparison.
Figure 26. Scatter plots of IL8-CEA Score by Disease Status

The median IL8-CEA was 0.42 with an interquartile range of 0.32-0.65. The distribution was non-normal upon Shapiro-Wilk testing (p<0.001). The IL8-CEA score increased with advancing disease status, from control (median 0.30, IQR 0.26-0.39), stage II (median 0.42, IQR 0.33-0.55) to stage IV (median 0.77, IQR 0.60-0.88), p<0.001 (Figure 26).
4.4 Discussion

In this study of sixty prospectively-enrolled participants, we observed distinct profiles of systemic inflammatory markers between healthy controls and colorectal cancer patients with stage II and stage IV disease. We found that IL-6, IL-8, CRP and CEA increased across the three groups. The widest ranges of values were observed in the stage IV group although metastatic site and number of organs involved did not clearly influence any of the markers studied. On ROC curve analysis, IL-8 and CEA were identified as the most effective discriminators between groups and when combined the resulting score outperformed any one marker.

Our finding that circulating IL-6, IL-8, CRP and CEA increased in association with disease progression corroborates other reports\(^ {298, 303, 395-398}\). In their study of 75 patients, Mahboob et al utilised Proseek and bioplex immunoassays to identify IL-8, CEA and Prolactin as the most stage-specific cytokines from their panel of 92 biomarkers \(^ {397}\). Olsen’s group measured plasma levels of 40 cytokines in 174 patients and found IL-8, as well as CCL20, CCL27 and MIF, to be associated with disease stage \(^ {303}\). Additionally, Grotowski et al reported significantly higher levels of serum CEA, IL-6 and IL-8 in patients with advanced stage disease compared to those with localised disease \(^ {398}\). Together, these findings suggest that systemic pathways involving inflammatory cytokines IL-6 and IL-8 appear to be activated not only in patients with overt malignant dissemination \(^ {382, 399}\) but also in those with macroscopically localised disease, albeit to a lesser degree. It is also suggested that serum CEA may be implicated in these pathways\(^ {398}\).

Inflammation within the primary tumour microenvironment is known to involve IL-6\(^ {216}\) and IL-8\(^ {400, 229}\), which act in an autocrine and paracrine manner on both tumour and
immune cells to promote tumour growth and progression\textsuperscript{193, 211}. Furthermore, the link between disease stage and systemic inflammation with CEA has been observed elsewhere\textsuperscript{298} and may be explained by mechanisms described by Lee & Lee and Beauchemin in their respective reviews \textsuperscript{401, 402}. Here the evidence is set out in favour of tumour-derived-CEA binding with hepatic Kupffer cells, resulting in their production of pro-inflammatory cytokines IL-6, IL-1\& and TNF\alpha\textsuperscript{403, 404}. Circulating levels of IL-1\& and TNF\alpha have been reported to rise in a stage-dependent manner\textsuperscript{193} and these two pro-inflammatory cytokines are known to induce IL-6 and IL-8 via the nuclear factor \textsuperscript{K}B (NF \textsuperscript{K}B) pathway\textsuperscript{204}. The link is further supported by Krystek-Korpacka et al’s finding that CEA levels positively correlated with rising IL-6 and IL-8\textsuperscript{405}. CEA release may therefore provide an important link between local and systemic inflammation.

IL6, IL8, CRP and CEA have been implicated, through experimental evidence, in a number of pro-metastatic systemic processes many of which are only beginning to be uncovered. In their review article, McAllister and Weinberg\textsuperscript{232} summate the experimental evidence for tumour-derived factors acting at distant sites to, i) prepare them for metastatic colonisation, ii) promote angiogenesis, and iii) promote the metastatic tumour microenvironment. The IL8-CEA combined score developed through the course of this study may reflect activity of these pro-metastatic systemic processes and be useful as a means of risk-stratifying patients. Although individually IL-8 and CEA have been shown to be associated with oncological outcome in colorectal cancer, no other studies have evaluated the prognostic value of a combined IL8-CEA score. This requires further evaluation in a longitudinal prospective study.
An association has been observed between inflammatory markers and a number of patient characteristics. One of the most important of these is advancing age. Franceschi et al coined the term, “inflammaging”, which refers to an observed increase in circulating levels of inflammatory markers in the elderly population. Age-related immune dysfunction is thought to underlie this as a cumulative result of life-long antigen exposure and repeated inflammatory stimuli. It is noteworthy that other reports, including the present study, have failed to demonstrate an association between inflammation and advancing age. Additionally, our present study did not corroborate others that have observed obesity, ethnicity and co-morbidities especially cardiovascular disease and diabetes, to be associated with circulating levels of inflammatory markers, particularly IL-6 and CRP. Our study may have been underpowered to detect these associations, although an association between CEA and patient ethnicity was observed in the present study (p=0.05) and has been reported by others. Therefore, patient characteristics may affect circulating levels of CEA and inflammatory cytokines and should be evaluated in future longitudinal studies as potential confounders of patient outcomes.

Limitations

Selection of Control subjects

Hospital policy meant that patients undergoing colonoscopy were fasted for a minimum of six hours prior to undergoing their procedure. Consequently, circulating biomarker levels may have been raised in these subjects due to dehydration and haemo-concentration. Additionally, patients recruited into stage II and stage IV groups were not routinely fasted prior to having their blood samples taken and this may have introduced measurement bias into the study. On the other hand, by selecting control subjects by this method allowed...
subjects with colorectal malignancy or inflammation to be definitively excluded from the group. Furthermore, patients attending hospital investigation under the suspicion of colorectal malignancy tended to be similar to patients with stage II and stage IV disease in terms of age and co-morbidity (Table 18). A truly healthy control population would have significantly varied with regard to these, potentially, confounding variables.

Additionally, patients with colorectal polyps were included in the healthy control group. In their study, Pengjun et al. 417 reported differences in the levels of IL-10, IL-8, MMP-2 and MMP-9 between patients with adenomas compared to those without and whilst this finding was acknowledged, our decision to include patients with polyps was to better reflect the general population, in whom colorectal polyps may be present 418-420. Furthermore, no patients were included into this group that had a polyposis syndrome or evidence of high-grade dysplasia, both of which are associated with an increased risk of malignant transformation.

Sample collection and processing

A high degree of inter-study variation is observed in circulating cytokine levels and this is likely to be due, at least in part, to a non-standardised approach to sample handling and cytokine quantification between studies 421-424. Many methodological variables are known to influence cytokine levels, including the anticoagulant used, duration of sample storage, number of freeze-thaw cycles and manufacturer of assay kits 425, 426. These factors may explain the discordant values we observed for IL-1β and TNFα compared to previous studies 421-424. However, in our study one manufacturer was used to detect all cytokines studied and stringent laboratory processes were followed in order to minimise inter-assay
variation \(^{425,426}\). Additionally, assay kits with suitable reference ranges were selected for use in the study.

**Radiological staging**

The current study was limited by its reliance on radiological staging to define patients with stage two rectal cancer. Whilst histopathological staging would provide a definitive evaluation, the results would be confounded by response to neo-adjuvant therapies. Our approach was more clinic-focused as staging was assigned based on data available to the treating clinical teams at the time of initial treatment decision-making.

**Correlation with local factors**

The extent to which raised circulating inflammatory biomarkers simply reflect inflammation within the primary tumour was not addressed in the present study. Galon and colleagues have shown that the immunological phenotype within the primary tumour has an important bearing on outcome, as demonstrated by their validated “immunoscore”\(^{162,163,186}\). Furthermore, an association between tissue IL-6 concentration and advancing TNM stage has been shown although has not been widely studied\(^{427}\).

Finally, Väyrynen et al attempted to evaluate the relationship between the immune cell density within the primary tumour and circulating inflammatory markers to suggest an association, although weak, may exist\(^{180,306}\). The markers measured in the present study are not tissue-specific and the primary tumour, as well as the liver and pre-metastatic or metastatic sites may have contributed to the circulating concentrations measured. On the other hand this may be an advantage in providing global evaluation of the host-response to cancer.
4.5 Conclusion

The results of this study suggest a systemic inflammatory disturbance may occur in colorectal cancer patients prior to overt disseminated disease. Whilst widely studied prognostic tools including the modified Glasgow prognostic score and neutrophil-lymphocyte ratio incorporate this principle, they rely on non–specific markers of inflammation. In the context of emerging evidence of both pro- and anti-tumour systemic inflammatory pathways, more specific markers or combinations of markers are needed to develop prognostic tools that are applicable in the clinical setting. Furthermore, we found that a combined score of IL-8 with CEA acted as an effective marker for systemic disease. A longitudinal study is required to determine whether these markers have prognostic value.
Chapter Five
Exploratory prospective study of
prognostic circulating biomarkers
5.1 Introduction

In Chapter Four, we found individual cytokines IL-6 and IL-8 to differ significantly between patients with localised and systemic disease. Furthermore, it has been put forward that a proportion of early recurrences in colorectal cancer are due to systemic micro-metastases present at the time of initial treatment\(^{428}\). Therefore, cytokine markers including IL-6 and IL-8 may be useful in predicting early recurrence by identifying patients with otherwise occult distant metastases.

We also found that combining IL-8 with CEA resulted in an improved sensitivity and specificity in identifying patients by disease status, compared to either marker alone. It is pertinent to also examine this combination in a prospective prognostic study. As CEA is the most widely studied circulating prognostic biomarker in colorectal cancer and one of the few if not only to be measured in everyday clinical practice, its prognostic value would provide a useful benchmark against which to compare more novel approaches.

Although a paucity of data was available in the literature, a multi-marker approach was found to show some prognostic value, based on the findings of our systematic review (Chapter Three). This is not surprising given the multi-faceted interaction between the tumour and host through a multitude of inflammatory pathways including both pro- and anti-tumour activity. In Chapter Three, we identified a shortfall of the included studies to be the study of very large panels of cytokines that were not justified through experimental or clinical evidence linking them to colorectal cancer progression or prognosis. Given the wide range of inflammatory cytokines, we selected a panel of eight cytokines with roles in colorectal cancer progression that have been observed experimentally. These cytokines, and their roles, are summarised in Table 21, below.
Table 21 Experimental data linking cytokines with colorectal cancer progression

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mechanisms of Action in Colorectal Cancer Progression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNFα</strong></td>
<td>Activates NF-kB in intestinal epithelial cells to promote proliferation and resistance to apoptosis</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>Induces EMT in CRC through activation of SNAI1</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>Triggers proliferation of CRC cell lines in a STAT3-dependent manner</td>
<td>390, 431</td>
</tr>
<tr>
<td></td>
<td>TNF blockade diminishes tumour development in AOM-DSS-treated mice</td>
<td>432</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Ifng−/− mice show more and larger intestinal tumours compared to wild-type</td>
<td>433</td>
</tr>
<tr>
<td></td>
<td>Stimulates anti-tumour immunity through CD8+ T-, NK cell- and macrophage-mediated cytotoxicity</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>Exogenous IFN-g inhibits growth of HT-29 CRC cells in mice. Inhibits EGFR/Erk1/2 and Wnt/B-catenin signaling pathways through induction of STAT1</td>
<td>435</td>
</tr>
<tr>
<td><strong>IL-2</strong></td>
<td>Anti-tumour effects through activation of NK cells</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>Systemic IL-2 therapy decreased tumour burden in mice with colonic adenocarcinoma (CC-36 cell line)</td>
<td>437</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>Promotes EMT of CRC cells through activation of STAT6 and loss of e-cadherin</td>
<td>438</td>
</tr>
<tr>
<td></td>
<td>Activates M2-like TAM polarization in CRC</td>
<td>439</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>IL6-dependent STAT3 signaling promotes CRC cell line DSS-AOM proliferation and survival</td>
<td>386, 440</td>
</tr>
<tr>
<td></td>
<td>Pro-tumourigenic effects observed through induction of Notch1 and CD44 expression</td>
<td>441</td>
</tr>
<tr>
<td></td>
<td>Induction of microsatellite instability in CRC cell lines</td>
<td>442</td>
</tr>
<tr>
<td></td>
<td>NF-KB-IL6-STAT3 pathways promotes CRC progression</td>
<td>443, 444</td>
</tr>
<tr>
<td></td>
<td>Induces EMT via JAK2/STAT pathway</td>
<td>445, 446</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>Promotes outgrowth, vascularity and metastasis of CRC xenografts</td>
<td>207</td>
</tr>
<tr>
<td></td>
<td>Induces tumourigenesis and tumour angiogenesis in DSS-AOM mouse CRC model</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td>Promotes cell proliferation, invasion and angiogenesis through activation of Akt and MAPK pathways</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Promotes EMT in CRC cell lines</td>
<td>210, 447</td>
</tr>
<tr>
<td></td>
<td>Addition to CRC cells suppresses apoptosis</td>
<td>448</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>Required in Treg cells to reduce tumour burden in ApcMin/+ mice</td>
<td>449</td>
</tr>
<tr>
<td></td>
<td>IL-10 deficient mice more susceptible to intestinal tumour development</td>
<td>450</td>
</tr>
<tr>
<td><strong>IL-17A</strong></td>
<td>Deficiency partially protects mice from CRC in ApcMin/+ and AOM-DSS models</td>
<td>451, 452</td>
</tr>
<tr>
<td></td>
<td>Promotes secretion of VEGF, inducing tumour angiogenesis</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>Facilitates cell cycle progression in CRC cell lines through increased expression of Sca-1</td>
<td>454</td>
</tr>
</tbody>
</table>

AOM-DSS: Azoxymethane and dextran sulfate sodium murine model of colitis-associated cancer
Aims

The aims of this chapter were:

❖ To evaluate baseline plasma IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, IFNϒ and TNFα as predictors of disease free survival in a cohort of patients undergoing treatment of colorectal cancer with curative intent

❖ To compare the above cytokines with clinico-pathological factors as predictors of disease free survival.

❖ To compare the prognostic value of a combined Cytokine-CEA score with CEA alone

5.2 Methods

Study design

This was a single-centre prospective observational cohort study.

Patient selection & recruitment

Consecutive patients with newly diagnosed stage I to III colorectal adenocarcinoma undergoing treatment with curative intent at Wellington Regional Hospital between October 2016 and June 2018 were eligible for recruitment in this study.

The exclusion criteria, ethics and plasma sample collection and storage were as described in Chapter Four (4.2). All of the stage II patients from the Chapter Four study were also included in this study in addition to those subsequently recruited up to the end of June 2018.
Clinical, radiological and histopathological data were collected prospectively for the cohort. This included demographic variables (age, sex, ethnicity, past medical history, drug history), and tumour characteristics (location, AJCC stage, perineural invasion, lymphovascular invasion, cell type, histological grade). Microsatellite instability (MSI) status was based upon histological evidence of deficient mismatch repair proteins MLH1, MLH2 or MSH. Data on the treatment received was also collected (neo-adjuvant therapy, adjuvant therapy).

Sample collection & Storage

Sample collection and storage was as described in Chapter Four (5.2).

Cytokine measurement.

Assays were performed using cytometric bead array (BD Biosciences) and were not carried out by the author. All assays, however, were carried out by or under the direct supervision of experienced University research staff.

Cytometric Bead Arrays (CBA)

A FACSCanto™ II plate loader-equipped flow cytometer (Becton Dickinson, USA) was used with BD FACSComp™ software and BD CaliBRITE™ beads (BD, Biosciences) for data acquisition and FCAP Array™ software was used for data analysis.

BD™ CBA Flex Sets (BD Biosciences) were used for IL-2 (Catalogue #558270) and TNFα (Catalogue #558273) with Human soluble protein master buffer kits (Catalogue #558265) (BD Biosciences). BD™ CBA enhanced sensitivity flex sets were used for IFN-γ (Catalogue #561515), IL-6 (Catalogue #561512), IL-8 (Catalogue #561513), IL-10 (Catalogue #561514), IL-17A (Catalogue #562143) and IL-4 (Catalogue #561510) with human enhanced
sensitivity master buffer kits (Catalogue #516523) (BD Biosciences). All assays were optimised so that each analyte had 300 bead events captured and were performed according to the manufacturer’s instructions.

**Clinical follow up & Endpoints**

Patients were followed up according to a standard protocol that was implemented by the Department of General Surgery at Wellington Regional Hospital in line with the New Zealand Guidelines Group document ‘management of early colorectal cancer’\(^\text{12}\). This included three to six monthly outpatient clinic assessments, yearly CT chest, abdomen and pelvis scans and three-monthly serum carcinoembryonic antigen measurement. Endpoint data was collected prospectively following the censor date of 1\(^{st}\) July 2019 in order to allow a minimum of 12 months follow-up for the cohort.

The endpoint measured was Disease Free Survival, defined as the time from surgery to either recurrence (local or distant) or death. Recurrence was by histological confirmation or following radiological assessment and multi-disciplinary team consensus opinion.

**Statistical analysis**

In addition to the statistical methods described in the Chapter Three, Receiver operator characteristic curves were used to identify the optimal cut-off value for individual cytokines. A cut off value of 3.0ng/mL was used for pre-operative CEA, based on the findings of Chapter Two. Kaplan-Meier curve analysis was performed using the Mantel-Cox log rank test in order to compare disease free survival by clinical-pathological variable and biomarker. A p-value <0.05 was taken to be statistically significant.
Ethics

All patients were recruited and samples collected and stored with ethical approval by Health and Disability Ethics Committee (‘Establishment of human tissue bank of surgical cancers for future unspecified research, ref: 15/CEN/143). Cytokine measurement was approved by the Health and Disability Ethics Committee (‘Molecular biomarkers in colorectal cancer’, ref: 18/CEN/138). The Ngai Tahu Research Consultation Committee were consulted during all stages of this study.
5.3 Results

174 patients were eligible for recruitment of which 101 patients were excluded leaving 73 patients included in the study. The reasons for exclusion are illustrated in Figure 27.

Figure 27. Flowchart of patient selection for prospective longitudinal study
Patient demographics

The patient demographics are shown in Table 2.2. The median age was 70 years, 49.3% were male and 87.7% of European ethnicity. Aspirin was taken by 17.8% of patients and another form of Non-steroidal anti-inflammatory drug (NSAID) was taken by a further 5.5%.

Table 2.2. Demographics of patients included in prospective longitudinal study

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>70</td>
<td>(60-77)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>(49.3)</td>
</tr>
<tr>
<td>Female</td>
<td>37</td>
<td>(50.7)</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>64</td>
<td>(87.7)</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>(12.3)</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>27.8</td>
<td>(5.5)</td>
</tr>
<tr>
<td><strong>Smoker</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(8.2)</td>
<td></td>
</tr>
<tr>
<td><strong>PMHx</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IHD</td>
<td>9</td>
<td>(12.3)</td>
</tr>
<tr>
<td>DM2</td>
<td>11</td>
<td>(15.1)</td>
</tr>
<tr>
<td>CVD</td>
<td>2</td>
<td>(2.7)</td>
</tr>
<tr>
<td>Renal</td>
<td>2</td>
<td>(2.7)</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>(2.7)</td>
</tr>
<tr>
<td><strong>DHx</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>13</td>
<td>(17.8)</td>
</tr>
<tr>
<td>Other NSAID</td>
<td>4</td>
<td>(5.5)</td>
</tr>
<tr>
<td>Insulin</td>
<td>2</td>
<td>(2.7)</td>
</tr>
<tr>
<td>Metformin</td>
<td>5</td>
<td>(6.8)</td>
</tr>
</tbody>
</table>

**Tumour Characteristics**

The cohort included 46 colonic cancers and 27 rectal cancers. According to TNM staging, there were 11 stage I, 30 stage II and 32 stage III patients (Table 23). Tumour characteristics by cytokine (Table A1) and associations between cytokines (Table A2) are shown in the Appendix.

Table 23. Tumour characteristics for patients included in prospective longitudinal study.

<table>
<thead>
<tr>
<th>Tumour Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Site</strong></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>29 (39.7)</td>
</tr>
<tr>
<td>Left colon</td>
<td>17 (23.3)</td>
</tr>
<tr>
<td>Rectum</td>
<td>27 (37.0)</td>
</tr>
<tr>
<td><strong>TNM Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11 (15.1)</td>
</tr>
<tr>
<td>II</td>
<td>30 (41.1)</td>
</tr>
<tr>
<td>III</td>
<td>32 (43.8)</td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>20 (29.0)</td>
</tr>
<tr>
<td>Low</td>
<td>49 (71.0)</td>
</tr>
<tr>
<td><strong>Mucinous</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8 (11.0)</td>
</tr>
<tr>
<td>No</td>
<td>65 (89.0)</td>
</tr>
<tr>
<td><strong>Perineural invasion</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (18.3)</td>
</tr>
<tr>
<td>No</td>
<td>58 (81.7)</td>
</tr>
<tr>
<td><strong>Lymphovascular invasion</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9 (12.5)</td>
</tr>
<tr>
<td>No</td>
<td>63 (87.5)</td>
</tr>
<tr>
<td><strong>Lymph node yield ≥12</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>49 (67.1)</td>
</tr>
<tr>
<td>No</td>
<td>24 (32.9)</td>
</tr>
<tr>
<td><strong>Microsatellite Instability</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (19.6)</td>
</tr>
<tr>
<td>No</td>
<td>45 (80.4)</td>
</tr>
</tbody>
</table>
Treatments

Neo-adjuvant therapy was administered in 17/27 (63.0%) of patients with rectal cancer. Ten out of 27 (37.0%) received short course radiotherapy and seven of 27 (25.9%) received long course chemo-radiation.

Adjuvant chemotherapy was recommended at multidisciplinary meeting in none of 11 patients with stage 1 disease, nine of 30 (30.0%) with stage II and 22 of 32 (68.8%) with stage III. Four out of nine (44.4%) stage II patients in whom adjuvant chemotherapy was recommended went on to complete treatment compared to 16/22 (72.7%) in stage III. Five patients declined chemotherapy, four patients developed toxic side effects and two developed a medical complication requiring early termination of treatment (one deep vein thrombosis and one myocardial infarction). Eight patients were not offered chemotherapy following clinic assessment by a medical oncologist.

Outcomes

The median duration of follow-up was 18 months with a range of 12-31 months. During this time there was one death (1.4%), which was cancer-related and occurred at 10 months in a patient who developed distant metastatic disease.

There were seven (9.58%) recurrences in total, including one local recurrence. The local recurrence was detected at the site of anastomosis at 20 months follow up in a patient who did not develop distant recurrence.

There were six (8.2%) distant recurrences detected at a median of 13 months, ranging from one to 22 months, of follow up. The liver and the peritoneum were the most common sites of recurrence with involvement in four patients each. Two patients had
more than one site involved, both of which were distant in each case. None of the patients had lung metastases.

**Plasma Cytokines**

Three out of the ten cytokines (IL-6, IL-8 and IL-10) resulted in detectable readings in at least 75% of the cohort (Table 24). The remaining cytokines were therefore excluded from further analysis. The lower limit of detection of the assay was used as the cytokine value in cases where levels fell beneath this.

Table 24. Number of patients with detectable levels and limits of detection, by cytokine assay

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Detectable Levels, N 73 (%)</th>
<th>Limit of Detection, fg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL8</td>
<td>71 (97.3)</td>
<td>69.9</td>
</tr>
<tr>
<td>IL6</td>
<td>60 (82.2)</td>
<td>68.4</td>
</tr>
<tr>
<td>IL10</td>
<td>60 (82.2)</td>
<td>13.7</td>
</tr>
<tr>
<td>IL17A</td>
<td>30 (41.1)</td>
<td>26.1</td>
</tr>
<tr>
<td>TNFα</td>
<td>6 (8.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>IL2</td>
<td>2 (2.7)</td>
<td>88.9</td>
</tr>
<tr>
<td>IL4</td>
<td>1 (1.3)</td>
<td>140</td>
</tr>
<tr>
<td>IFNY</td>
<td>0 (0)</td>
<td>14.8</td>
</tr>
</tbody>
</table>

IL: Interleukin, TNF: Tumour necrosis factor; IFN: Interferon

**IL-6**

The median IL-6 value was 2118.09 fg/mL (IQR, 318.52-3359.78). Using the Shapiro-Wilk test, the data was found not to be normally distributed (p<0.001). The skewness Z-score was 15.4 and Kurtosis Z-score 39.1.
IL-8

The median IL-8 value was 7020.23 fg/mL (IQR, 4074.46-10718.38). Using the Shapiro-Wilk test, the data was found not to be normally distributed (p<0.001). The Skewness z-score was 9.4 and Kurtosis Z-score 8.0.

IL-10

The median IL-10 value was 423.56 fg/mL (IQR, 76.42-778.65). Using the Shapiro-Wilk test, the data was found not to be normally distributed (p<0.001). The Skewness z-score was 16.3 and Kurtosis Z-score 46.2.

Receiver Operator Curve Analysis

On Receiver Operator Characteristic (ROC) curve analysis, IL-10 (AUC=0.66) was associated with the highest areas under the curve, although the AUCs were similar for all three individual cytokines (Table 25). An optimum cut-off value was selected for each of the markers and are presented in the table below along with their corresponding sensitivity and specificity. The ROC curves for individual cytokines IL6, IL8 and IL10 are shown in Figure 28.
Table 25. Receiver operator characteristic curve analysis for disease free survival, by circulating biomarker

<table>
<thead>
<tr>
<th></th>
<th>IL-6, fg/mL</th>
<th>IL-8, fg/mL</th>
<th>IL-10, fg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC</strong></td>
<td>0.65</td>
<td>0.63</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Cut off value</strong></td>
<td>2646.85</td>
<td>7112.04</td>
<td>151.59</td>
</tr>
<tr>
<td><strong>Sensitivity, %</strong></td>
<td>71.4</td>
<td>71.1</td>
<td>65.2</td>
</tr>
<tr>
<td><strong>Specificity, %</strong></td>
<td>65.2</td>
<td>54.5</td>
<td>85.7</td>
</tr>
</tbody>
</table>

IL: Interleukin; CEA: Carcinoembryonic antigen; CRP: C-reactive protein
Figure 28. Receiver Operator Characteristic curves for disease free survival, by cytokine

- IL-10
- IL-8
- IL-6

Sensitivity (%) vs. 100% - Specificity%
After cut-off points were determined using the above method, a high IL-6 was found to be associated with high histological grade (p=0.02) and microsatellite instability (p=0.01). IL-6 was strongly associated with IL-8 (p<0.001) but not IL-10 (p=0.58) (Appendix – Table A1. IL-10 was not associated with IL-6 (0.58) or IL-8 (p=0.60) (Appendix – Table A3).

**Survival Analysis**

Kaplan-Meier curve analyses were performed to evaluate the ability of each prognostic marker to predict disease free survival.

![Kaplan-Meier curves of disease free survival by TNM stage](image)

Figure 29. Kaplan-Meier curves of disease free survival by TNM stage

Although a trend toward decreasing DFS was observed with advancing stage, this was not found to be statistically significant (p=0.28) (Figure 29). However, mucinous (p=0.01), high-grade histology (p=0.04) and MSI (p=0.002) were significantly associated with a poorer DFS (Table 26).
Out of the three individual cytokines, only IL-10 (p=0.01) was associated with DFS, where a low IL-10 predicted poor DFS. IL-6 (p=0.06) and IL-8 (p=0.13) were not associated with DFS (Table 26, Figure 30).

**IL8-CEA Score**

Given that IL8-CEA was found to be an effective discriminator by disease status in Chapter Five, we evaluated its prognostic value in the present study. Patients with both a raised IL8 and CEA were given a score of ‘1’ and all others were scored ‘0’. This method was chosen in order to produce a dichotomous variable for simplicity and ease of subsequent analysis. Equal weighting was given to both variables due to a lack of data in the available literature comparing the effect size of IL8 and CEA in predicting DFS.

Disease Free Survival was 73.7% in those with an IL8-CEA score of ‘1’ compared to 96.3% in those with a score of ‘0’ (p=0.003) (Figure 30).

**IL10-CEA Score**

IL-10 was identified as the individual cytokine with the greatest ability to stratify patients into favourable and poor DFS in sample population. Therefore, it was combined with CEA in order to produce evaluate a combined IL10-CEA score. Given the inverse relationship between a high IL-10 and poor DFS, a combined score of ‘1’ was assigned for patients with both a low IL-10 and high CEA. All others were assigned a score of ‘0’.

Disease Free Survival was 68.5% in those with an IL10-CEA score ‘1’, compared to 96.5% in those with a score of ‘0’ (p=0.001). This showed some improvement on CEA alone, which was associated with a DFS of 83.8% when raised and 97.2% when not raised (p=0.03) (Figure 30).
Table 26 Disease Free Survival by Tumour Characteristic and Circulating Biomarker

<table>
<thead>
<tr>
<th></th>
<th>DFS (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNM Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>93.3</td>
<td>0.28</td>
</tr>
<tr>
<td>III</td>
<td>84.4</td>
<td></td>
</tr>
<tr>
<td><strong>Mucinous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>62.5</td>
<td>0.01</td>
</tr>
<tr>
<td>No</td>
<td>93.8</td>
<td></td>
</tr>
<tr>
<td><strong>High Grade</strong></td>
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Figure 30. Kaplan-Meier curves of disease free survival (DFS), by circulating biomarker.
5.4 Discussion

This exploratory study of 73 prospectively recruited patients, found low IL-10 to be an indicator of poorer disease free survival in stage I-III colorectal cancer after a median follow-up duration of 18 months. We found that combining this cytokine marker with CEA appeared to improve upon the prognostic value of CEA alone.

Our observation of an inverse relationship between high IL-10 and distant recurrence has been reported, although not consistently, in the published literature \(^{291, 455, 456}\). A meta-analysis by Zhao et al included 1788 patients from 21 studies and concluded that a raised baseline IL-10 was associated with poorer overall and disease-free survival\(^ {455}\) across numerous cancer types. However, only 30 patients with colorectal cancer were included in this meta-analysis and it is uncertain whether the authors’ conclusions can be generalised to this cancer type \(^ {291}\). Abtahi et al observed a “dual association” of serum IL-10 and colorectal cancer whereby IL-10 levels were lower in CRC patients than in controls but higher in CRC patients with poor prognosis \(^ {456}\). The validity of their study is similarly limited by its small sample size of 30 subjects but whose findings nevertheless reflect experimental data on the role of IL-10 in CRC, which is similarly opposing. That is to say, evidence exists both of a protective role of IL-10 by inhibiting cancer-enabling inflammation as well as a tumourigenic role through inhibition of anti-tumour immunity\(^ {456}\). Huang et al’s \textit{in vivo} study on human melanoma cell line A375P in mice demonstrated anti-tumour and anti-metastatic activity of IL-10 \(^ {457}\). Such properties were demonstrated by Birgisson et al who, in their study of 261 colorectal cancer patients, found recurrence free survival to be shorter in patients with low IL-10\(^ {458}\). These results corroborate those of our own although the effect of IL-10 on time to recurrence was not
statistically significant on Cox regression univariate analysis (p=0.07). The potentially “dual role” of IL-10 in colorectal cancer highlights the importance of interpreting IL-10 levels in combination with other markers.

In Chapter Four, we found that an IL8-CEA score was associated with the presence of metastatic disease. In addition to this, the IL8-CEA, and IL10-CEA, scores in the present study showed some association with DFS on Kaplan-Meier curve analysis. The combined score may act as a marker for occult metastases present at the time of surgery, or alternatively, indicate pre-metastatic niche formation. In their CRC mouse model, Seubert and colleagues showed that pre-metastatic niche formation within the liver was in part dependent on neutrophil chemotaxis \(^{459}\), which in turn is known to be a main function of IL-8 \(^{388}\). Additionally, Shao et al demonstrated the release of IL-6 from Kupffer cells upon the arrival of exosomes derived from primary CRC cells prior to their dissemination\(^{460}\).

However, the short follow-up duration in the present study make pre-metastatic niche formation a less likely mechanism of the recurrences observed than occult metastases that were already present.

We observed a poorer DFS in patients with both a raised IL-8 and CEA, compared to a raised CEA alone. This observation may indicate a functional role for the CEA molecule in systemic inflammatory pathways. Such a role has been demonstrated experimentally by Holmer and colleagues in their *in vitro* study whereby colon cell lines incubated with IL-6 were found to upregulate expression of CEA-related cell adhesion molecules CEACAM5 and CEACAM6 \(^{461}\). Furthermore, Wagner et al found that human colon cancer cell lines expressing high levels of CEA were much more likely to metastasise to the liver of mice than those expressing low levels \(^{462}\). Other *in vitro* \(^{391}\) and *in vivo* \(^{463}\) studies have shown
activation of Kupffer cells by CEA-binding to be accompanied by inflammatory cytokine release, including IL-6\textsuperscript{464}. A further advantage of utilising a combined Cytokine-CEA score is to offset the paradox that poorly differentiated colorectal cancers, whilst known to be associated with poorer prognosis\textsuperscript{465}, are less likely to have preserved CEA expression\textsuperscript{343}.

**Limitations**

The current study has a number of limitations to acknowledge.

Primarily, the sample size and particularly the number of events, seven, meant that this study was significantly underpowered. This is apparent by the unexpected finding that TNM stage was not predictive of DFS although a trend toward worsening DFS by advancing stage was observed, particularly in stage III patients compared to stage I and II. Given the low number of events it is possible that by dividing the cohort into three groups by TNM stage diminished the statistical power, beyond other analyses of dichotomous variables. As the prognostic value of TNM stage in predicting DFS is well-established, this finding calls into question the validity of the other findings of this study.

Furthermore, a general rule-of-thumb for regression analysis is the “rule of ten”. That is, one predictive variable should be studied per every ten events in order to minimise overfitting\textsuperscript{466}. As a minimum for this, two variables, TNM stage and a single circulating biomarker, would be included as independent variables in a multi-variate regression analysis, requiring approximately twenty events. In Chapter Two, we found a recurrence rate of 53/227 (23.3\%) in stage I to III patients over five years of follow-up. Therefore, between 80 and 90 patients would have had to be recruited and followed up for five years. This is a conservative estimate given that the study in Chapter Two included
emergency presentations, which are associated with poorer outcomes. The inclusion of other prognostic variables such as histological grade, lymphovascular and perineural invasion and MSI status in multi-variate regression analysis would further increase the number of required events. However, achieving such a sample size and follow-up duration was not feasible given the time- and resource-constraints of the present study. As part of this exploratory study, Kaplan-Meier curves were utilised to compare DFS by a single variable at a time. However, this meant that the association between a circulating biomarker and DFS was not adjusted for any of the other established predictors of DFS, including TNM stage and thus vulnerable to confounding.

Further to this, the study sample was heterogeneous in terms of a number of important factors including tumour location (colonic and rectal), nodal involvement and the administration of neo-adjuvant and adjuvant therapies. As a result, aspects of tumour biology such as responsiveness to systemic therapies may have further confounded the results of this study. In order to address this, sub-group analysis would be necessary.

Baseline CEA results were visible to treating clinicians during this study and may have introduced bias. The pre-operative CEA could have influenced the multi-disciplinary team’s decision to diagnose metastatic disease on staging imaging, or recurrence on surveillance imaging. Blinding the clinical teams to pre-operative CEA values would not be feasible or ethical given that it makes up part of the standard of care. Additionally, patients with a raised pre-operative may have been followed up more closely although data from the present study in insufficient to confirm or refute this. Additional study into the compliance with surveillance investigations and follow-up was outside the scope of this study but would have allowed for this form of bias to be evaluated.
A possible anomaly of the present study is the finding that none of the recurrences observed in the present study occurred in the lungs. This is in contrast to our findings in Chapter Two, where the lung was found to be the second most common site of metastasis after the liver. In their population-based study of 5671 colorectal cancer patients undergoing treatment with curative intent, van Gestel et al found 20% of lung metastases to occur within one year, compared to 40% of liver metastases\textsuperscript{465}. The longer time-to-recurrence reported with lung metastases may have meant that with a relatively short follow-up duration in the present study lung metastatic recurrences were effectively selected out.

An additional limitation to this study is the equal weighting given to the components of the combined (IL8-CEA and IL10-CEA) scores, despite it being unlikely that their effect size on DFS is exactly equal. This could be addressed in a larger, sufficiently powered, study by performing Cox proportional regression. The hazard ratio could be calculated for IL8, IL10 and for CEA based on univariate Cox regression, as predictors of DFS, and taken to be the effect size of each marker. IL8, IL10 and CEA could then be appropriately weighted according to their respective effect sizes to produce the combined score. This score would then need to be evaluated in subsequent prospective studies in order to be externally validated.
5.5 Conclusion

In this exploratory prospective study, we found IL-10 and CEA to be indicators of disease free survival among 73 patients with stage I-III colorectal cancer undergoing treatment with curative intent, after a median follow up duration of 18 months.

Combined IL8-CEA and IL10-CEA scores appeared to improve upon CEA alone in predicting disease free survival. Although the study was underpowered, as a proof-of-concept, it suggests there may be an added benefit to combining circulating inflammatory cytokine measurement with baseline CEA to prognosticate patients with colorectal cancer. This is a novel approach and requires further investigation to test this hypothesis using multivariate regression and sub-group analysis in a large prospective longitudinal study with sufficiently longer follow up duration.
Chapter Six

Summary
6.1 Summary

Colorectal cancer is a major cause of cancer-related mortality and, despite recent advances in treatment, the five-year recurrence rate following surgery is around 20%\textsuperscript{323,335}. The ability to stratify patients according to risk of recurrence is critical in guiding therapeutic management decisions and historically has relied on the TNM stage and other tumour characteristics. However, the host-response is becoming increasingly recognised as playing an influential role on clinical outcomes. In turn, circulating biomarkers may offer a means by which tumour-host interactions are evaluated.

The aims of this thesis stem from a growing body of evidence linking systemic inflammation to disease outcome through a number of proposed mechanisms and these are described in Chapter One. Ultimately, we set out to investigate whether circulating inflammatory cytokines provide prognostic value in potentially curable colorectal cancer.

Chapter Two includes a retrospective cohort study including patients undergoing treatment with curative intent at a single institution between 2010 and 2012. We evaluated the pattern of disease recurrence in 237 consecutive patients, of whom 24.9% developed recurrence at a median follow up of 61 months. We found that distant recurrences made up the majority (74.6%) of those observed and that 72.9% of all recurrences were observed in the first two years of follow-up with annual recurrence rates diminishing significantly thereafter. Such early distant metastases may represent a sub-set of patients in whom occult systemic disease is present at the time of surgery. In turn, circulating biomarkers may provide a means of identifying patients with otherwise occult disease. Next, we evaluated the prognostic value of pre-operative carcinoembryonic antigen. Although widely available and considered the most used tumour marker in
clinical practice, we found only 58.6% of patients to have data available for baseline CEA; the remaining patients were therefore excluded. We found that with an optimum cut-off value of 3.0ng/mL, CEA had a sensitivity of 70% and a specificity of 52% in predicting disease free survival. In contrast to a number of existing studies, which use 5.0ng/mL as the cut-off, this finding suggests there may be prognostic value from a high-normal pre-operative CEA. On multi-variate analysis, pre-treatment CEA was found to be an independent predictor of disease free survival (HR 1.99, 95% CI 1.07-3.69, p=0.03) and overall survival (HR 3.17, 95% CI 1.46-6.89, p=0.004). On the basis of these findings, CEA was established as a benchmark against which other, more novel, circulating biomarkers could be compared in future studies. The practices and outcomes we observed in Chapter Two were comparable to those of other contemporary cohorts in the published literature. This meant future studies at the same institution, as part of this thesis, were unlikely to be subject to biases from anomalous clinical practices or outcomes.

In Chapter Three, we turned to circulating inflammatory cytokines. Through experimental evidence, a number of cytokines have been implicated in pro-metastatic systemic pathways. Studies have shown circulating levels of individual cytokines IL-6, TNFα and IL-1β among others to be prognostic but few have undertaken a multi-marker approach. Chapter Three was a systematic review of the literature evaluating the prognostic value of multiple cytokine analysis in colorectal cancer. Seven studies were included in the review, which excluded studies evaluating two or fewer cytokines. Five studies included patients with stage I-IV disease; the remaining two studies included stage IV disease only. Only two studies were prospective in design and three of the seven studies reported disease free survival as an endpoint of interest. Sample sizes were
generally small, ranging from 46 to 205 and we found study quality to be poor to moderate, based on the risk of bias across six domains. Additionally, there was a high degree of variability in the size and contents of the cytokine panels tested. Four studies tested panels of ten or greater cytokines using pre-manufactured panels and failed to cite evidence justifying many of the cytokines tested. Four studies utilised a combined cytokine score and some promise was shown of this approach, although variation existed in the cytokines included and criteria used to create the score. The results of the systematic review did not yield a panel of candidate cytokine markers for further study partly due to the high number and wide range of cytokines studied. However, the review did inform our approach of selecting a small panel of cytokines justified through experimental data of a link with colorectal cancer progression.

In Chapter Four, participants were prospectively recruited into three groups. Plasma cytokines IL-6, IL-8, IL-1β and TNFα were compared across the groups: healthy control subjects (n=19), stage II (n=22) and stage IV (n=19). For a biomarker to be prognostic, we would expect it to differ between these three disease states. Based on the findings of Chapter Two, pre-treatment CEA was included in the study for comparison. TNFα and IL-1β levels were detectable in fewer than 75% of the cohort and were therefore excluded from further analysis. We found IL-6 (p=0.005), IL-8 (p<0.001) and CEA (p<0.001) to increase significantly with advancing disease status and a combined ‘IL8-CEA score’ was marginally more stage-specific than either marker alone (p<0.0001). IL-6, IL-8 and CEA were among those studied further in Chapter Five. This was an exploratory study of a panel of cytokines for evaluation as prognostic markers, by means of a longitudinal observational cohort study.
Here, 73 patients with newly diagnosed non-metastatic colorectal cancer were prospectively recruited at a single institution. Baseline plasma samples were collected and subsequently analysed by CBA for a panel of eight cytokines. This panel was selected on the basis of experimental evidence linking each cytokine to CRC progression. This included IL-6 and IL-8 from the previous study in addition to TNFα, IFNY, IL-2, IL-4, IL-10 and IL-17A. Among the eight cytokines tested, IL-6, IL-8 and IL-10 yielded recordable values in greater than 75% of the cohort and the remaining five cytokines were excluded from further analysis. Patients were followed up to a minimum of 12 months post-operatively. The median follow up duration was 18 months (range, 12-31 months) and the disease free survival was 90.4%. Individually, CEA (p=0.03) and IL-10 (p=0.01) were found to be predictive of disease free survival on Kaplan-Meier survival curve analysis. Following the findings of Chapter Four, whereby the combining of IL-8 and CEA improved upon CEA alone in differentiating disease status, we evaluated a combined IL8-CEA score in this study as well as IL10-CEA, given that IL-10 was identified as a potential prognostic marker through Kaplan-Meier curve analysis. We found that both scores appeared to improve marginally upon CEA alone in identifying a sub-set of patients with poorer disease free survival. However, the study sample size and follow up duration precluded any multivariate survival analysis or sub-group analysis. Therefore, it was not possible to conclude whether a raised IL8-CEA or IL10-CEA score is an independent prognostic factor for poor prognosis in colorectal cancer. Instead, the results identify IL-8 and IL-10 as potential candidates for future evaluation through longitudinal prognostic studies, and suggest a possible additional prognostic value of combining cytokine measurement with pre-operative CEA.
Limitations

There were a number of overall limitations of the studies included in this thesis to acknowledge.

Firstly, the clinical studies were all carried out at a single clinical institution. This may in turn impact the generalisability of our findings to other centres and patient populations. In order to address this problem, we explored the option of extending patient recruitment to a second hospital. As well as broadening patient participation this would have also increased the sample size and with it statistical power. However, due to distance from the University laboratory, there would have been an unacceptable delay to processing. Specifically, our target of centrifuging all blood samples within thirty minutes would not have been possible and consequently the decision was made to confine recruitment to the host institution so not to compromise the quality of stored plasma samples. In addition to these resource-constraints, the time-constraints of this thesis ultimately prevented our ability to perform a comprehensive prognostic longitudinal study. The study described in Chapter Five was significantly underpowered for such an analysis, which would require a five-year follow-up duration and a much larger sample size. These measures would improve the statistical power, and allow a multi-variate Cox proportional hazard model to be utilised. This study design would also allow for other prognostic factors such as TNM stage, histological grade, lymphovascular and perineural invasion to be adjusted for.

The problem of over-fitting is relevant to the studies included in this work whereby an optimal cut-off value was determined for a variable in the same data set that it was evaluated in. Examples of this include the pre-operative CEA in Chapter Two, and circulating cytokine markers in Chapter Five. The post hoc selection of an optimal cut-off
may have over-estimated the true effect of the measured variable on outcome. A measure in which to address this would be to externally validate these same cut-off points in an independent study sample.

Although the TNM stage is widely acknowledged as the most reliable and strongest predictor of prognosis in colorectal cancer, it was not identified as an independent prognostic predictor in Chapter Two, when only patients with available CEA data were analysed. Although the sample size and therefore the statistical power was significantly reduced in the second part of the study by excluding patients with missing CEA data, one would still expect TNM stage to be a stronger prognostic predictor than pre-operative CEA, as has been found elsewhere in the literature. Similarly, TNM stage failed to be observed as a significant predictor of DFS on Kaplan-Meier survival curve analysis in Chapter Five. It is possible that the findings were confounded by the stage-dependency of treatment regimes, such as the greater likelihood of patients with Stage III to receive adjuvant chemotherapy, compared with Stage II disease. Nevertheless, the results are discordant with the existing available literature and this must be considered when drawing other conclusions from the study.

It must be noted that the conclusions of this thesis regarding the prognostic role of inflammatory cytokines cannot be generalised to emergency presentations. This applies to up to 20% of all CRC presentations \(^\text{46}\). Emergency patients, especially those presenting with perforation, are likely to exhibit grossly deranged inflammatory cytokine levels as a consequence of their pathology. In contrast, changes in the baseline inflammatory state that we observe as a reflection of the host-response to cancer are likely to be
comparatively subtle. Therefore, emergency presentations were excluded to prevent skewed data and an impaired ability to assess the host-response to cancer.

An improvement to the present study would be the post-operative measurement of CEA and cytokine values. There is evidence that post-operative biomarker levels may be more prognostic than pre-operative values. For example, Konishi et al observed a similar prognosis in patients with a normal pre-treatment CEA to those with a raised pre-treatment CEA that normalised after surgery. The difficulty with applying the same approach and measuring post-operative plasma cytokine levels is in selecting an appropriate post-operative time-point. A number of factors in the peri-operative timeframe could influence plasma cytokine levels including the operative approach, duration of surgery and the incidence of post-operative complications especially infective or inflammatory complications, such as anastomotic leak. Whether post-operative samples should be taken before or after adjuvant therapy would also have an impact on the results, particularly in the presence of toxic side effects such as colitis.

6.2 Future Directions

The findings of this thesis suggest that circulating inflammatory cytokines may provide additional prognostic value to CEA alone. The next step would be for the IL10-CEA score, developed through this body of work, to be externally validated in a large prospective cohort study. This requires a significantly larger sample size and a follow up duration of five years to provide sufficient statistical power. Furthermore, multivariate analysis or subgroup analysis would be necessary to account for confounding variables, including TNM stage. Additionally, it is important to investigate the association between systemic inflammatory status and DFS among patients in whom adjuvant chemotherapy is not
administered. Such studies would include stage 1 and low-risk stage II patients only and therefore require significantly larger sample sizes due to the lower distant recurrence rate than those observed in our studies. Were a combined IL10-CEA score to be externally validated as a predictor of DFS, particularly in patients who would not ordinarily be offered adjuvant chemotherapy, there could be scope for further study into the benefit of adjuvant chemotherapy in such individuals.

The link between systemic inflammation and outcome in colorectal cancer has led to interest into the therapeutic benefits of anti-inflammatory agents. Although the mechanism remains unclear, a number of retrospective observational studies have suggested Aspirin use to be associated with reduced CRC mortality\textsuperscript{468}. Furthermore, there is some indication that COX-2\textsuperscript{469} and PIK3CA\textsuperscript{470} may act as predictive biomarkers of a survival benefit of adjuvant Aspirin use. The findings of our thesis further strengthen the link between systemic inflammation and prognosis in colorectal cancer and future work should be carried out in the form of large prospective studies to investigate the potentially beneficial role of adjuvant Aspirin or NSAIDs in CRC.

Monoclonal antibodies that target specific cytokines have also been investigated in the treatment of solid tumours with some benefit \textsuperscript{471,472}. For instance, Siltuximab, an anti-IL6 agent, was shown in a phase I/II clinical trial to stabilise disease in more than 50% of patients with progressive metastatic renal cell cancer\textsuperscript{473}. Currently, no clinical benefit has been seen from Siltuximab in patients with advanced colorectal cancer\textsuperscript{474} although the evidence is limited to a single phase I/II trial. Some have suggested that targeting a single cytokine is unlikely to be beneficial when multiple cytokines are known to act as part of a network\textsuperscript{471}. This argument is strengthened by the findings of this thesis whereby multiple
cytokines have been shown to influence outcome. Combination therapies with multiple cytokine targets have been proposed and based on our findings, IL-8 is an additional potential target. A phase I trial of HuMax-IL8, an anti-IL8 monoclonal antibody, has shown the drug to be safe and well-tolerated in fifteen patients with solid organ tumours, including four patients with colorectal cancer\textsuperscript{475}. 

In addition to targeting individual cytokines, there may be a role for downstream targets, such as that of the JAK/STAT pathway \textsuperscript{476}. A phase II clinical trial showed that a possible improvement in survival of patients with metastatic pancreatic adenocarcinoma when JAK1/2 inhibitor Ruxolitinib was used in combination with capecitabine\textsuperscript{477}. Specifically, this benefit was seen only in a sub-group of patients with elevated systemic inflammatory markers CRP or mGPS. This may suggest a role for scores such as IL10-CEA in patient selection for novel anti-inflammatory therapeutic agents and this warrants future investigation.
Appendix
Table A 1. Tumour Characteristics by IL-6, IL-8 and IL-10

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