AN INVESTIGATION INTO THE RELATIONSHIP BETWEEN THE TUMOUR AND ITS ENVIRONMENT AND SURVIVAL IN PATIENTS WITH OPERABLE COLORECTAL CANCER

BY

James H. Park

BSc MBChB MRCS (Gla)

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPOPHY (PhD)

TO

THE UNIVERSITY OF GLASGOW

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

Abstract

Colorectal cancer is the second most common cause of cancer death in the Western World. Although staging and prognosis is presently based on pathological assessment of primary tumour invasion and the presence of lymph node and distant metastases, it is increasingly recognised that other factors pertaining to both the tumour and host may similarly affect outcome. The local and systemic environment, encompassing host inflammatory responses and the tumour microenvironment, are examples of such. However, how such measures may compliment present TNM-based staging are not clear. Furthermore, tumour and host factors, both modifiable and non-modifiable, which may determine the local and systemic environment, remain to be fully determined.

The present thesis examined the clinical and prognostic utility of assessment of the local and systemic environment, and potential tumour and host factors which may determine these responses. The following conclusions were drawn:

Examining patients from the United Kingdom and Japan, Chapter 2 and 3 concluded that assessment of the systemic inflammatory response, utilising the modified Glasgow Prognostic Score, provides further prognostic stratification in addition to TNM stage. Although the proportion of patients exhibiting an elevated systemic inflammatory response differed between populations, the prognostic value was comparable.

Chapter 4 validated assessment of the tumour stroma percentage as a prognostic factor independent of TNM stage and the local inflammatory cell infiltrate (cancer-specific survival HR 1.84, 95% CI 1.17-2.92, P=0.009). Chapter 7 further confirmed the prognostic value of a combined tumour microenvironment score, based on assessment of the generalised inflammatory cell infiltrate and tumour stroma percentage, in patients with primary operable colorectal cancer. This score, termed the Glasgow Microenvironment Score, was able to stratify patients into a good prognostic group, with five-year survival of

89%, an intermediate group with a two-fold increased risk of death and five-year survival of 75%, and a poor prognostic group, with a four-fold increased risk of death and five-year survival of 51%.

Chapters 5 and 6 identified the presence of mismatch repair deficiency and activation of the JAK/STAT3 as two potential mechanisms which may determine host local and systemic inflammatory responses. However, the prognostic value of such candidate mechanisms was weak, suggesting that other pathways and tumour characteristics are implicated, and that molecular heterogeneity is likely to play an important role in determining not only the local and systemic environment, but also outcome.

Chapter 9 concluded that the Immunoscore, an immunohistochemistry-based assessment of T-lymphocyte density within the tumour microenvironment, held greater prognostic value than assessment of the generalised inflammatory cell infiltrate using the Klintrup-Mäkinen grade. However, assessment of tumour stroma percentage provided additional prognostic value irrespective of the methodology employed to examine the local inflammatory cell infiltrate. Furthermore, the results of Chapters 7, 8 and 9 together suggested that loss of the local, anti-tumour immune infiltrate was the primary event which allows continued tumour growth, development of a tumour-supportive microenvironment and propagation of a systemic inflammatory response.

Chapter 10 concluded that pre-diagnosis use of aspirin but not statins was associated with a lower modified Glasgow Prognostic Score, despite strong associations with comorbidity and BMI. This did not translate into an improvement in survival, potentially reflecting the underlying indication for use of these drugs primarily as cardiovascular secondary prevention medications.

Finally, Chapter 11 examined the clinical utility of assessment of the tumour microenvironment using colonoscopic biopsy specimens, concluding that the use of biopsy-derived specimens was feasible. Furthermore, in addition to identifying patients who may benefit from therapies targeting the tumour microenvironment, assessment of a biopsy-derived Glasgow Microenvironment Score had comparable prognostic value to full section assessment of the tumour microenvironment.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I am sincerely grateful for the assistance provided by the following individuals, each of whom has offered their expertise, encouragement and guidance throughout the period of research working towards this thesis:

Dr Campbell Roxburgh	Academic Unit of Surgery, Glasgow Royal Infirmary
Professor Donald McMillan	Academic Unit of Surgery, Glasgow Royal Infirmary
Professor Paul Horgan	Academic Unit of Surgery, Glasgow Royal Infirmary
Dr Joanne Edwards	Institute of Cancer Sciences, University of Glasgow

Declaration

The work presented in this thesis was undertaken during a period of research between 2012 and 2015 in the Academic Unit of Surgery, Glasgow Royal Infirmary. This work has been completed whilst working as a Specialist Trainee Registrar in General Surgery in the West of Scotland deanery between 2015 and 2016.

I declare that I have performed the following work:

- Validation of clinicopathological data, including markers of systemic inflammation and survival data for patients included in all Chapters
- Statistical analyses in all Chapters
- Assessment of TSP for all patients (Chapters 4/5/6/7/8/9/11)
- KM grading of the local inflammatory cell infiltrate for a proportion of patients not previously scored (Chapters 4/5/6/7/8/9)
- Co-scoring of MMR status for 25% of cases (Chapter 5/6/7/8/9)
- Optimisation of immunohistochemical staining for STAT3 and pSTAT3, and subsequent staining of tissue microarray (Chapter 6)
- Scoring of cases for STAT3 and pSTAT3 expression (Chapter 6)
- Collection of pre-operative prescribing data, ASA grade and BMI for majority of cases (Chapter 10)
- Retrieval of archival biopsy specimens (Chapter 11)
- Scoring of biopsy TSP and T-lymphocyte density, and optimisation of automated scoring (Chapter 11)
- Prospective data collection for departmental colorectal cancer database

The following work was undertaken by colleagues:

- Data from Dokkyo Medical University, Japan, was collected and provided by Professor Ishizuka, Dokkyo Medical University (Chapter 3)
- Co-scoring of 10% of cases for TSP was performed by Campbell Roxburgh (Chapters 4/5/6/7/8/9/11)

- The majority of KM grading of the local inflammatory cell infiltrate has previously been performed by Campbell Roxburgh (Chapters 4/5/6/7/8/9)

- Necrosis has previously been performed by Colin Richards (Chapters 4/8)

- Immunohistochemical staining and grading of T-lymphocyte subsets has previously been performed by Colin Richards (Chapters 4/5/6/8/9/11)

- Immunohistochemical staining for MMR proteins was performed by Clare Orange (Chapter 5/6/7/8/9)

- Determining MMR status was performed in the majority of cases by Arfon Powell (Chapter 5/6/7/8/9)

- Co-scoring of 10% of cases for STAT3 expression was performed by Jennifer Clark (Chapter 6)

- Pre-operative prescribing data was collected for some cases by Shou Kee Ng (Chapter 10)

- Immunohistochemical staining for T-lymphocytes in colonoscopic biopsies was performed by Clare Orange (Chapter 11)

- Co-scoring of T-lymphocyte density and TSP in colonoscopic biopsies was performed by Campbell Roxburgh (Chapter 11)

Publications

The work presented in this thesis has resulted in the following publications:

The impact of anti-inflammatory agents on the outcome of patients with colorectal cancer.
Park JH, McMillan DC, Horgan PG, Roxburgh CS
Cancer Treatment Reviews 2014 Feb; 40(1): 68-77

The relationship between tumour stroma percentage, the tumour microenvironment and survival in patients with primary operable colorectal cancer.
Park JH, Richards CH, McMillan DC, Horgan PG, Roxburgh CSD
Annals of Oncology 2014 Mar; 25(3): 644-651

Comment on 'Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis'
Park JH, Roxburgh CSD, McMillan DC
British Journal of Cancer 2014 Dec; 111(12): 2372

Evaluation of a tumour microenvironment-based prognostic score in primary operable colorectal cancer.
Park JH, McMillan, DC, Powell AG, Richards CH, Horgan PG, Edwards J, Roxburgh CSD
Clinical Cancer Research 2015 Feb; 21(4): 882-8

Colorectal cancer, systemic inflammation, and outcome: staging the tumor and staging the host.
Park JH, Watt DG, Roxburgh CSD, Horgan PG, McMillan DC
Annals of Surgery 2016 Feb; 263(2): 326-36

Mismatch repair status in patients with primary operable colorectal cancer: associations with the local and systemic tumour environment.
Park JH, Powell AG, Roxburgh CSD, Horgan PG, McMillan DC, Edwards J.
British Journal of Cancer 2016 Mar; 114(5): 562-70

Comparison of the prognostic value of measures of the tumour inflammatory cell infiltrate and tumor-associated stroma in patients with primary operable colorectal cancer.
Park JH, McMillan DC, Edwards J, Horgan PG, Roxburgh CSD.
Oncoimmunology 2016; 5(3): e1098801

Signal transduction and activator of transcription-3 (STAT3) in patients with colorectal cancer: associations with the phenotypic features of the tumour and host.
Park JH, van Wyk H, McMillan DC, Quinn J, Clark J, Roxburgh CSD, Horgan PG, Edwards J.
Clinical Cancer Research 2017; 23(7):1698-1709

Tumour invasiveness, the local and systemic environment and the basis of staging systems in colorectal cancer.
Park JH, van Wyk, H, Roxburgh CSD, Horgan PG, Edwards J, McMillan DC.
British Journal of Cancer 2017; 116:1444-50

Presentations

The work presented in this thesis has resulted in the following presentations:

Pre-diagnosis aspirin and statin use up-regulates the local inflammatory response in colorectal cancer: implications for neodajuvant treatment.
Digestive Diseases Week, Florida, USA, 2013 (Best of DDW Poster Tour, Poster of Distinction)

The association between tumour stroma, tumour pathology and survival in patients with primary operable colorectal cancer.
National Cancer Research Institute, Liverpool, UK 2013 (Poster)

Host inflammatory responses, tumor stroma percentage and survival in colorectal cancer.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2014 (Poster)

The relationship between tumor and host factors and survival in patients undergoing adjuvant chemotherapy for colorectal cancer.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2014 (Poster)

Mismatch repair status, host local and systemic inflammatory responses and survival in patients with primary operable colorectal cancer.
Digestive Diseases Week, Chicago, USA, 2014 (Best of DDW Poster Tour, Poster of Distinction)

Mismatch repair status, inflammation and outcome in patients with primary operable colorectal cancer.
Association of Coloproctologists of Great Britain & Ireland Tripartite Meeting, Birmingham, UK, 2014 (Oral)

The Glasgow Prognostic Score and survival in colorectal cancer: experience in 1000 consecutive patients undergoing curative resection.
National Cancer Research Institute, Liverpool, UK, 2014 (Poster)

Inflammation and survival in 1000 consecutive patients with colorectal cancer.
West of Scotland Surgical Association, Glasgow, UK, 2014 (Poster prize winner)

The clinical utility of a tumour microenvironment-based histopathological score in patients with primary operable colorectal cancer.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2015 (Poster)

Assessment of the tumor inflammatory cell infiltrate in pre-operative colonoscopic biopsies of patients with primary operable colorectal cancer.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2015 (Poster)

The relationship between pre-diagnosis use of aspirin and statins and tumour and host characteristics of patients with colorectal cancer.
Association of Surgeons of Great Britain & Ireland, Manchester, UK, 2015 (Oral)

Pre-operative assessment of the tumour inflammatory cell infiltrate using colonoscopic biopsies of patients with primary operable colon cancer.
Association of Surgeons of Great Britain & Ireland, Manchester, UK, 2015 (Oral)

The relationship between the tumour microenvironment, early metastases and survival of patients with primary operable colon cancer.
Association of Surgeons of Great Britain & Ireland, Manchester, UK, 2015 (Oral)

The clinical utility of the modified Glasgow Prognostic Score in 1000 consecutive patients undergoing potentially curative colorectal cancer resection.
Cell Symposia: Cancer, Inflammation, and Immunity, Sitges, Spain, 2015 (Poster)

A novel tumor microenvironment-based prognostic score, the Glasgow Microenvironment Score, in patients with primary operable colorectal cancer.
Cell Symposia: Cancer, Inflammation, and Immunity, Sitges, Spain, 2015 (Poster)

The local inflammatory response in colorectal cancer – what does it mean?
Royal Society of Medicine Coloproctology Study Day: Colorectal Cancer and The Host, London, UK, 2015 (Invited Lecture)

Signal transduction and activator of transcription 3 (STAT3), host inflammatory responses and survival of patients with colorectal cancer.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2016 (Poster)

Tumor site, clinicopathological characteristics and survival of patients undergoing primary elective colorectal cancer resection.
American Society of Clinical Oncology Gastrointestinal Cancer Symposium, San Francisco, USA, 2016 (Poster)

Staging the tumour and staging the host in primary operable colorectal cancer: East and West.
American Society of Clinical Oncology, Chicago, USA 2016 (Poster)

Definitions/Abbreviations

5-FU – 5-fluorouracil

AJCC – American Joint Committee on Cancer

APC – adenomatous polyposis coli

APER – abdominoperineal resection

ASA – American Society of Anaesthesiologists

ASCO – American Society of Clinical Oncologists

AUC – area under the curve

BMI – body mass index

BMP – bone morphogenic protein

CEA – carcinembryonic antigen

CI – confidence interval

CIMP – CpG island methylator phenotype

CIN – chromosomal instability

CLR – Crohn's-like lymphoid reaction

COX-2 – cyclo-oxygenase-2

COXIB – cyclo-oxygenase-2 inhibitors

CRP – C-reactive protein

CSS – cancer-specific survival

CT – computed tomography

DMU – Dokkyo Medical University

DPX - distrene, plasticizer and xylene

EMT – epithelial-mesenchymal transition

EUS – endoluminal ultrasound

FAP – familial adenomatous polyposis

GMS – Glasgow Microenvironment Score

GPS – Glasgow Prognostic Score

GRI – Glasgow Royal Infirmary

H2RA – histamine-2 receptor antagonist

H&E – haematoxylin and eosin

HNPCC – hereditary nonpolyposis coli

HR – hazard ratio

IHC – immunohistochemistry

IL – interleukin

JAK – Janus kinase

KM – Klintrup-Mäkinen

LNR – lymph node ratio

mGPS – modified Glasgow Prognostic Score

MHC – major histocompatibility complex

MMR – mismatch repair

MRI – magnetic resonance imaging

MSI – microsatellite instability

MSS – microsatellite stable

NF-κB – nuclear factor kappa-B

NLR – neutrophil: lymphocyte ratio

NPS – neutrophil platelet score

OR – odds ratio

OS – overall survival

PET – positron emission tomography

PGE_2 – prostaglandin E_2

PI3K – phosphatidylinositol 3-kinase

PLR – platelet: lymphocyte ratio

ROC – receiver operating characteristics

SCPRT – short course preoperative radiotherapy

SNP – single nucleotide polymorphism

STAT – signal transduction and activator of transcription

TGF*B* – transforming growth factor-*B*

TMA - tissue microarray

TME – total mesorectal excision

TNM – tumour, node, metastasis

TSP – tumour stroma percentage

UICC – Union for International Cancer Control

VEGF – vascular endothelial growth factor

Dedication

To Kirsty, my wife and best friend. For all your patience and support.

To my Mother, who has always believed in me.

Summary

Colorectal cancer is common, with over 1 million cases each year globally. Although advances in staging, surgical technique and chemotherapeutics have led to improvements in survival, approximately half of patients undergoing potentially curative resection die within five years. Currently, staging, prognosis and need for adjuvant treatment is based on pathological assessment of the tumour using the TNM staging system. In addition, other high-risk tumour characteristics, such as venous invasion, may predict increased risk of recurrence. It is clear however that current staging is inadequate and may fail to stratify risk effectively. As such, there is a need to identify other tumour and host characteristics which may be used to determine prognosis and guide treatment.

One such approach is assessment of the local and systemic environment, encompassing amongst other things the host inflammatory response and the tumour microenvironment. It is recognised that the presence of a conspicuous inflammatory cell infiltrate is a good prognostic factor independent of TNM staging. Several measures of the local inflammatory response have been proposed; whereas the Klintrup-Mäkinen grade provides a measure of the generalised inflammatory infiltrate, the Immunoscore is a more detailed measure of the predominantly cytotoxic T-lymphocytic response. Conversely, elevated systemic inflammatory responses, as measured by acute phase reactants and differential white cell count, are associated with poorer survival. One such systemic inflammatory response-based score, the modified Glasgow Prognostic Score, has been validated and reported internationally as a stage-independent marker of poor prognosis.

However, how such measures may be utilised in combination with current staging is not clear. In addition, how other components of the tumour microenvironment, such as the tumour-associated stroma, may relate to the local and systemic inflammatory response, and outcome, is unknown. Tumour and host characteristics, including anti-inflammatory drug

use, which may determine the local and systemic environment have not been fully determined. Finally, the feasibility of pre-operative assessment of the tumour microenvironment has yet to be established. The aim of the present thesis was to examine these questions.

Chapter 2 examined the prognostic value of combined assessment of the modified Glasgow Prognostic Score and TNM stage in patients with non-metastatic colorectal cancer. Using this combination, it was possible to identify patients with lymph node negative disease with poorer survival than those with lymph node positive disease. Furthermore, in patients with stage III colon cancer, it was possible to identify patients less likely to benefit from adjuvant chemotherapy.

Chapter 3 examined the prognostic value of the modified Glasgow Prognostic Score in two cohorts of patients from the United Kingdom and Japan. When compared to a cohort of patients from Japan, it was found that patients from United Kingdom were more likely to be systemically inflamed, even after controlling for clinicopathological characteristics determined to be associated with the presence of a systemic inflammatory response. Of interest however, the modified Glasgow Prognostic Score had similar prognostic value in both cohorts.

In Chapter 4, the relationship between tumour stroma percentage, other components of the tumour microenvironment and survival was examined. Although inversely associated with the local inflammatory cell infiltrate, tumour stroma percentage remained an independent prognostic factor, suggesting that it may be of complimentary value to measures of the local inflammatory cell infiltrate.

In Chapter 5 and 6, the relationship between mismatch repair status, STAT3 expression and the local and systemic environment were examined. It was found that both local and

systemic inflammatory responses were associated with mismatch repair deficiency, although both remained independently associated with survival irrespective of mismatch repair status. STAT3 expression was associated with loss of the local inflammatory cell infiltrate and elevated systemic inflammatory responses, however STAT3 itself was not independently associated with survival. These results again highlight the complex nature and multitude of factors underpinning the local and systemic environment.

Chapter 7 examined the prognostic value of a novel tumour microenvironment-based score, encompassing the Klintrup-Mäkinen grade and tumour stroma percentage. This score, termed the Glasgow Microenvironment Score was able to stratify survival greater than either measure alone. Furthermore, the associations between the components of this score suggest that it is loss of the local anti-tumour immune response which allows subsequent development and expansion of a tumour-supporting stroma.

Chapter 8 found that increasing tumour invasiveness, as measured by T stage, was associated with the development of a pro-tumour local and systemic environment. Similar to the results of Chapter 6, it was found that loss of the local inflammatory cell infiltrate preceded tumour stroma expansion and development of a systemic inflammatory response, again suggesting that loss of the local immune response is an important driver of disease progression. Furthermore, it was found that such measures at both a local and systemic level have similar if not greater prognostic value compared to current lymph node-based staging.

In Chapter 9, two different measures of the local inflammatory cell infiltrate, the Immunoscore and Klintrup-Mäkinen grade, were compared. It was found that the Immunoscore had greater fidelity with respect to determining prognosis of patients. Despite this, the addition of tumour stroma percentage still stratified survival greater than

either measure alone, again supporting the rationale for a comprehensive assessment of the tumour microenvironment as opposed to assessment of individual components alone.

In Chapter 10, it was found that aspirin but not statin use was associated with lower levels of systemic inflammation at time of diagnosis. Aspirin use was however not associated with improved survival. As these drugs were primarily prescribed for cardiovascular secondary prevention, it is likely that any oncological benefit in this patient group will be underestimated due to the high level of comorbidity associated with aspirin and statin use.

Chapter 11 examined the clinical utility of pre-operative assessment of the tumour microenvironment utilising colonoscopic biopsies. Both the local inflammatory cell infiltrate and tumour stroma percentage could be measured using biopsy specimens, with diagnostic accuracy of the former improved by the use of digital automated pathology. Furthermore, a derived, biopsy-based Glasgow Microenvironment Score had similar prognostic value to more conventional, full section based assessment of the tumour microenvironment in patients with colorectal cancer.

1 Introduction

1.1 Epidemiology of colorectal cancer

Colorectal cancer is common, with an estimated 1.2 million cases worldwide in 2008 (1, 2). Globally, it is the second most common cancer in females with over 570,000 cases in 2008, and the third most common cancer in males with over 663,000 cases. It is more common in developed countries, with the highest incident rates in Europe, Australia and New Zealand and Northern America (1). Although incidence is generally lower in developing countries, particularly Africa and South-Central Asia, rates of colorectal cancer are increasing in countries with historically low incidence rates. In 2008 there was an estimated 608,700 deaths from colorectal cancer (1).

In 2008, colorectal cancer was the most common cancer across Europe, with an estimated 436,000 incident cases, accounting for 13.6% of all cases of cancer (2). In 2009, it was estimated that the economic cost of colorectal cancer across the European Union was over EU €13 billion (3). The incidence of colorectal cancer in Europe has increased over the past two decades, particularly in males and in Central Europe (4).

In the United Kingdom, colorectal cancer is the fourth most common cancer overall, with 37,600 cases registered in 2008 (2). It is the third most common cancer in females behind breast and lung cancer, and the second most common cancer in males behind prostate cancer. Similar to much of the rest of Europe, the incidence of colorectal cancer has increased over past decades (5), however even across the United Kingdom, there is significant geographical variation in the incidence of colorectal cancer, with the highest rates observed in Scotland and Northern Ireland and the lowest rates in England. Whereas the age-standardised incidence rate across the United Kingdom is 47 cases per 100,000, this varies from approximately 53 cases per 100,000 in Scotland and Northern Ireland to 46 cases per 100,000 in England (5). Furthermore, incident rates for colorectal cancer are

greatest amongst more deprived areas, predominantly due to an increased incidence rate amongst males (6).

The incidence of colorectal cancer also varies by sex and age. Males have a higher incidence of colorectal cancer, predominantly due to an increase in the incidence of rectal cancers. In 2012, females accounted for 18,160 registered cases of colorectal cancer with an age-standardised incidence rate of 37 cases per 100,00, compared to 22,600 cases and an age-standardised rate of 56 cases per 100,000 in males (7). Similar disparity in temporal trends for colorectal cancer incidence also exist; whereas the European age-standardised incidence rate for males has increased by 29% between 1975-1977 and 2009-2011, it has only increased by 7% in females over the same period (5). In addition, the incidence of colorectal cancer is strongly associated with age, with 95% of colorectal cancers in the three years up to 2011 diagnosed in patients aged 50 and over (5). Although between the ages of 45 and 85 the incidence of colorectal cancer is greater in males than females, above the age of 85 the incidence is greater in females due to the larger, at risk, female population.

Overall, colorectal cancer is the second most common cause of cancer death in the United Kingdom (8). It is the third most common cause of cancer death in females, with 7,470 deaths in 2012 and an age-standardised mortality rate of 14 deaths per 100,000; similarly it is the third most common cause of cancer death in males, with 8,730 deaths in 2012 and a rate of 21 deaths per 100,000 (7). Consistent with incidence, there is geographical variation in colorectal cancer mortality rates across the United Kingdom, with higher mortality rates in Scotland for both males and females compared to England (8); whereas the European age-standardised mortality rate across the United Kingdom is 16.3 per 100,000, this varies from 19.2 to 15.9 deaths per 100,000 population in Scotland and England respectively. Deprivation also influences mortality from colorectal cancer, with a 30% higher mortality for males and 15% higher mortality for females from deprived areas

(8). Mortality rates have improved over time, with an overall decrease since the 1970s (8). In the ten years leading up to 2012 for example, the mortality rate amongst females decreased by 15% and for males decreased by 12%.

1.2 Colorectal carcinogenesis

Rather than being one distinct entity, colorectal cancer is a heterogeneous disease arising from a number of different molecular and genetic pathways. A prerequisite of carcinogenesis is the development of genetic instability, which is critical for the rapid accumulation of mutations required for cancer to develop (9). The chromosomal instability pathway, described by Vogelstein and Fearon in 1988 (10), is now thought to be too simplistic and may only describe carcinogenesis in around 85% of cases of colorectal cancer. Indeed, it is now recognised that at least three distinct molecular subtypes resulting in genetic instability exist; the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI), and the CpG island methylation pathway (CIMP). Although in reality these pathways may co-exist in some cancers (11), they remain a useful backbone for the molecular characterisation of patients with colorectal cancer.

1.2.1 Chromosomal instability pathway

Invasive colorectal carcinoma develops from non-invasive, precursor adenomatous polyps, with an accumulation of specific mutations occurring in tandem with this pathway (Figure 1.1). This traditional 'adenoma-carcinoma sequence' as described by Vogelstein and Fearon, is characterised by aneuploidy and a number of common mutations which appear to occur at specific time points in the transition from adenoma to carcinoma (12, 13). For example, loss of the *adenomatous polyposis coli (APC)* gene, either through mutation or loss of chromosome 5q, appears to occur at an early stage and can be observed in as many as 80% of early adenomata (14). In addition to the deleterious effects of loss of its tumour suppressor function, *APC* loss also disrupts mitosis further contributing to CIN (15). In contrast, loss of the tumour suppressor gene *p53*, usually through loss of chromosome 17p, is often a late event heralding the transition from a non-invasive to invasive lesion. Indeed, whereas *p53* loss may only be observed in less than a quarter of early adenomata, it has been observed in between 50-75% of colorectal carcinomas (13). Intermediary steps have

been described by Vogelstein, including *KRAS* mutations and loss of chromosome 18q. It has however been observed that these steps may not always occur in the same order, and indeed in some cases may not occur at all (9). Although the above 'traditional pathway' does not fully explain the complexity of carcinogenesis arising through the CIN pathway, it is nonetheless thought that approximately 80% of colorectal cancers develop through this mechanism.

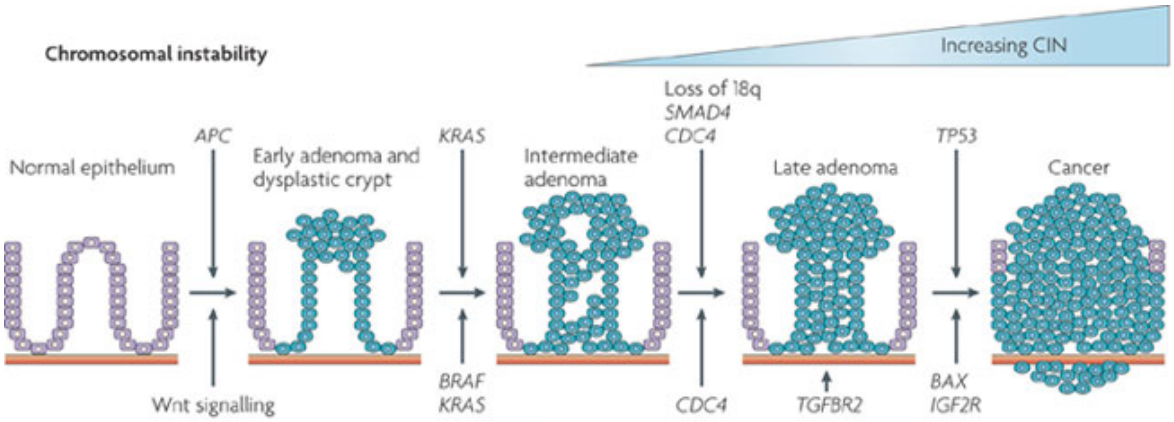


Figure 1.1 The adenoma-carcinoma sequence in sporadic colorectal cancer, displaying key molecular and genetic events which occur in the transition from non-invasive lesion to invasive cancer. Adapted from Walther, Johnstone et al. (11)

1.2.2 Microsatellite instability pathway

By the mid 1990s, it was recognised that up to 20% of tumours, including both hereditary and sporadic, arose via the MSI, or mutator, pathway (16). The molecular hallmark of tumours arising through this pathway is loss of function of the mismatch repair (MMR) protein machinery which normally rectifies DNA replication errors. These frameshift mutations commonly occur in repetitive nucleotide sequences known as microsatellites, many of which exist within key genes associated with carcinogenesis. Loss of MMR protein function may be a result of germline mutations, as observed in Hereditary Non-Polyposis Colorectal Cancer (HNPCC) or Lynch syndrome, where *hMSH2* and *hMLH1* are the most commonly affected genes. Approximately 15% of sporadic colorectal cancers may also arise through MSI, which usually occurs through epigenetic silencing of *hMLH1*, in turn leading to mutations within other MMR protein encoding genes (9).

Tumours arising through the MSI pathway display a distinct phenotype; they are predominantly right-sided, poorly differentiated or displaying mucinous differentiation, and are often considered less likely to metastasise (17-19). Furthermore, the tumour microenvironment of MSI tumours is often replete with tumour infiltrating lymphocytes (20). Such characteristics may be used to identify tumours which should be further assessed for MSI.

Detection of MSI may be performed by examining for instability within a panel of two mononucleotide (BAT25, BAT26) and three dinucleotide microsatellites (D2S123, D5S346, D17S250) known as the Bethesda panel (21). Tumours are defined as MSI if mutations are identified in at least two of the specified sites, and defined as microsatellite stable (MSS) if no mutations are identified. Tumours with a mutation within one site are termed as MSI-low, and are often categorised alongside MSS tumours, however may represent another distinct molecular entity. As testing for MSI is expensive, screening for loss of mismatch repair proteins using immunohistochemistry (IHC) may be an acceptable alternative (22), with tumours found to be MMR deficient considered for subsequent genetic sequencing for MSI.

1.2.3 CpG island methylation pathway

Epigenetic silencing of gene transcription by DNA methylation is an important homeostatic mechanism. It has been associated with the development of sporadic colorectal cancer in approximately 20% of cases (23), with hypermethylation of cytosine and guanine dinucleotide base pairs within gene promoter regions effectively 'switching off' tumour suppressor genes (9). Identification of CIMP tumours can be performed through assessment of the methylation status of specific gene promoter regions (24). Hypermethylation is commonly associated with a number of other molecular characteristics, such as mutated *BRAF* status, and has been shown to overlap with the MSI

pathway, with hypermethylation of *hMLH1* the driver event in the majority of sporadic MSI cases (25).

Similar to the MSI pathway, CIMP-associated tumours display typical phenotypic characteristics, including proximal location and an improved prognosis (11). Furthermore, whereas the precursor lesion in CIN and MSI-associated colorectal cancer is the adenomatous polyp, CIMP colorectal cancer is thought to arise from serrated hyperplastic polyps (23).

1.3 Aetiology of colorectal cancer

Colorectal cancer develops through an accumulation of genetic mutations over a prolonged period of time. Approximately 80% of cases are sporadic, developing through a complex interaction of environmental, host and genetic factors. Although 20% of patients will have a family history of colorectal cancer in a first-degree relative, an underlying inherited genetic condition is only identifiable in around 5% of all cases (26-28).

1.3.1 Environmental factors

It is now appreciated that environment plays an important role in the development of sporadic colorectal cancer. The role of environmental factors in colorectal cancer aetiology is supported by the vast differences in disease incidence across different geographical regions (29). In addition, migrants from regions with a low incidence of colorectal cancer moving to regions with a higher incidence experience an increase in risk within a generation (30, 31). Many of these factors, such as diet and sedentary lifestyle, are commonly perceived as part of Western culture; adoption of such traits in transitioning nations is thought to be responsible for the sharp rise in incidence in these countries (29, 32).

Dietary fibre

Burkitt first hypothesised an association between low dietary fibre intake and cancer risk in 1971 (33). The protective effect of dietary fibre is likely to be multifactorial, and in part may be due to decreased colonic transit time and dilution of carcinogenic compounds as a result of increased stool bulk (34). Furthermore, fermentation of dietary starch to butyrate and other short-chain fatty acids may promote cell cycle arrest, apoptosis and differentiation of colonocytes (35).

The results of prospective cohort studies suggest a protective effect of fibre on colorectal cancer incidence. The EPIC study, which included over 500,000 individuals over ten European countries, identified a 21% reduction in risk of colorectal cancer in those with the highest intake of dietary fibre compared to those with the lowest after controlling for folate intake (36). Further follow-up of the EPIC study cohort, with a mean follow-up of 11 years and accrual of 4,517 cases of colorectal cancer, further confirmed the protective benefit of dietary fibre, with an estimated 13% reduction in risk for every 10g increase in daily fibre intake (37). Subsequent meta-analysis of prospective observational studies, performed in association with the Continuous Update Project of the World Cancer Research Fund, has confirmed a similar dose-response relationship, particularly for fibre derived from cereals and whole grains (38).

Red and processed meat

The Second Expert Report of the World Cancer Research Fund recognised that red meat and processed meat (preserved by smoking, curing, salting or chemical preservation) was associated with a significant increase in the risk of colorectal cancer (39). The underlying mechanism is likely multifactorial, but involves exposure to carcinogenic compounds following cooking at high temperatures as well as following digestion. Furthermore, free iron from haem can promote free radical synthesis.

Following the Second Expert Report, additional prospective studies have further confirmed the association between both red and processed meats and colorectal cancer risk (40). A recent meta-analysis of ten prospective studies found a relative increase in risk of 17% for every 100g/day of red meat and an increase in risk of 18% for every 50g/day of processed meat consumed (40).

Calcium and Vitamin D

Increasingly calcium supplementation and, to a lesser extent, vitamin D supplementation, has been recognised as potentially protective against risk of colorectal cancer (41). Although this may reflect the importance of calcium as an intracellular second messenger and its role in the homoeostasis of proliferation and apoptosis, it has been hypothesised that the protective effects may be mediated by attenuation of tumour-associated inflammation (42). Given their inextricable association, it is difficult to ascertain the independent effect of calcium and vitamin D supplementation on colorectal cancer risk, however it is likely that any effect of vitamin D is mediated by the protective effects of calcium (41). Similarly, the perceived benefit of milk is likely due to dietary calcium supplementation.

Alcohol

Alcohol intake is associated with increased colorectal cancer risk. The EPIC study estimated an 8% increase in risk for every 15g/day intake (36). A differential effect may exist, with a higher risk for beer compared to wine. Furthermore, the risk may be greater in males, however this may be a surrogate for greater alcohol intake and choice of beverage (39). The relationship between alcohol intake and colorectal cancer risk displays a U-shaped curve, as it has previously been reported that moderate alcohol intake may reduce risk of colorectal cancer (42)

1.3.2 Host Factors

Age

Age is the biggest single risk factor for the development of colorectal cancer, with 95% of cancers diagnosed in patients over the age 50 (5). Increasing age allows for prolonged exposure to environmental and host risk factors. Furthermore, older age is associated with

telomere attrition and the accumulation of epigenetic changes, both of which predispose to increased mutational burden and carcinogenesis (43, 44).

Smoking

Smoking is associated with an increased risk of colorectal adenoma and carcinoma (45-48). Follow-up from the Nurses' Health Study and Health Professionals Follow-up Study suggested a 50% increased risk in females and almost two-fold increase in risk in males of colorectal cancer following a 35-year history of smoking, indicating the prolonged time taken for adenoma and carcinoma development (45, 46).

Physical activity

An increasingly sedentary lifestyle is a contributory factor to a number of common non-communicable diseases, and physical inactivity is thought to be a causative factor in as much as 10% of the worldwide burden of colorectal cancer (49). A meta-analysis of 21 studies found a reduction of approximately 25% in the risk of both proximal and distal colon cancer in the most physically active subjects when compared to the least active (50), however it is unclear if physical activity reduces the risk of rectal cancer (41). An increase in both recreational and total (i.e. occupational) physical activity has been found to be beneficial. An increase in total physical activity to the equivalent of 5 metabolic equivalent tasks for an hour per day (comparable to moderate intensity gardening), is associated with a 3% reduction in risk of colorectal cancer (41). The effect of physical activity on colorectal cancer risk is likely to be multifactorial; in addition to favourable effects on weight, body composition and gut motility, exercise also regulates insulin sensitivity and immune function.

Inflammatory bowel disease

Colorectal cancer risk is increased in the presence of longstanding inflammatory bowel disease (IBD), accounting for approximately 1% of all reported cases (51). The presence of ulcerative colitis is associated with a five to ten-fold increase in risk of colorectal cancer in comparison to age-matched controls (52), and risk is associated with cumulative exposure to colonic inflammation; whereas risk of colorectal cancer is 2% after 10 years duration of colitis, this increases to 18% following 30 years (51). Patients with Crohn's disease affecting the colon have a similar risk of colorectal cancer to those with ulcerative colitis (52).

Given the association between increased duration of mucosal inflammation and colorectal cancer risk, it is thought that chronic inflammation is the predominant driver of carcinogenesis in patients with IBD-associated colorectal cancer (53), with several key inflammatory pathways implicated in the development of colitis-associated colorectal cancer (54). Although a similar mutational burden can be seen in sporadic and IBD-associated colorectal cancer, the timing of these events is often altered, with *p53* mutations often occurring at a much earlier stage than in sporadic cancer (53).

Given the increased risk of colorectal cancer, it is advised that patients with IBD undergo colonoscopic surveillance for neoplastic disease. An initial colonoscopy should be offered after 10 years of disease activity (55), with the interval of subsequent examinations determined by risk stratification (53). Colonoscopic surveillance identifies patients with colorectal cancer at an earlier disease stage, however it is unclear what benefit this may ultimately have on survival, with any perceived benefit presumed to be secondary to lead-time bias (52). More recent studies however have suggested a survival benefit for routine colonoscopic screening of patients with IBD (56).

Obesity and the metabolic syndrome

Obesity is associated with increasing risk of colorectal neoplasia, including cancer. Whereas overall body fatness is associated with increasing risk (2% increase in risk per kg/m^2 (41)), abdominal fatness is associated with an even greater risk, reflecting the higher metabolic activity of visceral fat (41). The relationship between obesity and colorectal cancer risk is multifactorial, and in part reflects the pro-inflammatory state associated with obesity as well as the effect of adipocytes on sex hormone production.

Visceral obesity is also recognised as a component of the metabolic syndrome, a spectrum of metabolic and physiological risk factors including obesity, insulin resistance, hypertension, and atherogenic dyslipidaemia (57, 58). The metabolic syndrome is associated with increased risk of colorectal cancer, primarily through growth-promoting effects of insulin resistance and insulin-like growth factor-1 secretion, and the propagation of a pro-inflammatory state.

Cardiovascular disease

Patients with cardiovascular disease are at increased risk of colorectal neoplasia, including cancer, when compared to the general population. In addition to patients with symptomatic coronary and peripheral arterial disease (59, 60), it has also been shown that the detection of asymptomatic, screen-detected coronary artery disease also confers increased risk of colorectal neoplasia (61). Although this is likely due to shared risk factors for both diseases, the chronic inflammatory state associated with cardiovascular disease may also predispose to development of colorectal cancer.

Systemic inflammation

The presence of a chronic systemic inflammatory response is associated with increased risk of a number of cancers, including colorectal cancer. Prolonged exposure to a chronic

inflammatory response may lead to tissue infiltration by innate immune cells, and predispose to cellular and DNA damage through the production of reactive nitrogen and oxygen species (62). Furthermore, activated pro-inflammatory signalling pathways, such as nuclear factor κ-beta (NF-κB) are implicated in carcinogenesis (62). For this reason, it is not surprising that elevated concentrations of acute phase reactants, such as serum C-reactive protein (CRP) and inflammatory cytokines, has been associated with increasing risk of colorectal adenomata and cancer (63-65). Furthermore, elevated serum cytokine concentrations may also predict response to chemoprophylactic agents such as aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) (64).

Aspirin and non-steroidal anti-inflammatory drug use

Early evidence of a prophylactic effect of aspirin and NSAIDs in colorectal cancer originally arose out of studies of hereditary cancer syndromes. The use of NSAIDs decreases the number and size of colonic polyps in patients with familial adenomatous polyposis (FAP) (66). Similarly, aspirin also confers a protective effect on the colorectum in patients with Lynch syndrome (67). Over the past two decades, accumulating evidence from epidemiological studies has identified a potential role in the prophylaxis of sporadic disease, with an approximate 30% risk reduction with aspirin and non-aspirin NSAIDs and a potentially greater reduction with cyclooxygenase-2 inhibitor (COXIB) use (68, 69). A duration-dependent increase in risk reduction has been observed, with the greatest benefit seen after at least 10 years of continuous use. Cessation of regular use results in a return to normal population risk for subsequent colorectal cancer development. Secondary analyses of cardiovascular secondary prevention trials have found a significant benefit with aspirin doses commonly employed for cardiovascular disease prevention, rather than doses commonly associated with analgesic use (70). Despite such convincing evidence, concerns regarding the safety profile of NSAIDs have discouraged their use as prophylactic agents in the general population, at least until the optimal target population is identified (71).

Statin use

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins, are primarily used in the treatment of hypercholesterolaemia and atherosclerotic cardiovascular disease and are known to have a number of pleiotropic effects on cell proliferation, angiogenesis, inflammation and endothelial cell function (72, 73). Although a reduction in the risk of several cancers has been found in epidemiological studies (74-76), the results of meta-analyses suggest only a modest effect, if any, on colorectal cancer incidence in the general population (73, 77). Despite this, the results of *in* vivo studies and evidence of increased expression of HMG-CoA reductase in tumours arising from the left colon suggests a potential role for statins in the treatment of colorectal cancer (78).

Reproductive history and hormone replacement therapy

Although colorectal cancer is not considered hormone receptive, population level data has suggested a relationship between sex hormones and colorectal cancer risk. Increased lifetime exposure to endogenous oestrogen increases risk of colorectal cancer in postmenopausal women (79). Despite this, meta-analysis of data from observational studies suggests a reduced risk with both exogenous oestrogen and oestrogen-progestogen therapy (80). The protective effect may be limited to distinct molecular subtypes, and in particular risk of MSS, CIMP-negative and *BRAF*-wildtype tumours (81). Any potential benefit in reducing colorectal cancer risk may likely be offset by the increased risk of breast cancer with exogenous hormone therapy (80). Furthermore, results of a randomised, placebo-controlled trial (Women's Health Initiative) have suggested no clear benefit with combined hormone therapy, with a reduction in colorectal cancer incidence but increased disease stage at diagnosis in users of combined hormone treatment (82).

1.3.3 Hereditary colorectal cancer

Familial Adenomatous Polyposis

Familial adenomatous polyposis is associated with the development of hundreds of adenomatous polyps throughout the colon and rectum. The incidence is 1:5000 to 1:10000, accounting for less than 1% of all colorectal cancers (28), Patients with classic FAP have a 100% lifetime risk of developing colorectal cancer, often by the end of the fourth decade of life (26). Prophylactic colectomy is recommended when the affected carrier is in their teenage years. In addition to colorectal polyps and cancer, FAP is associated with a high incidence of extracolonic tumours including duodenal and desmoid tumours, which are the second and third most common causes of death in those patients who have undergone prophylactic colectomy (83).

Familial adenomatous polyposis displays an autosomal dominant pattern of inheritance, caused by germline mutations in the tumour suppressor *APC* gene. Attenuated FAP has been identified as a separate polyposis syndrome, with affected patients exhibiting fewer polyps throughout the colon and a reduced, albeit still high, lifetime risk of colorectal cancer (28). Whereas the underlying mutation in FAP results in a non-functioning protein, patients with attenuated FAP express a mutated but functioning form of APC. A further polyposis syndrome with no germline *APC* mutation and a mutation within *MUTYH* has also been identified (28). An autosomal recessive disorder, patients with MUTYH-FAP have a similar polyp burden to those with attenuated FAP and a lower lifetime risk of colorectal cancer.

Hereditary Non-Polyposis Colorectal Cancer

In the 1960s, Henry Lynch described a ‘cancer family syndrome’, with an increased predisposition to a number of cancers but primarily those affecting the colon and rectum (84). The syndrome, known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC), or

Lynch syndrome, has an incidence of approximately 1:1000 in the general population, and is the most common familial colorectal cancer syndrome, accounting for around 2-5% of all cases (85). Affected carriers are at an increased risk of colorectal cancer in addition to stomach, small bowel, biliary, urothelial, ovarian, endometrial, skin appendage and brain tumours. Compared to patients with sporadic colorectal cancer, those with Lynch syndrome have a younger age of onset, with diagnosis often in the fifth decade of life. Inheritance is autosomal dominant, with penetrance of around 80%, however this varies with the underlying germline mutation (26). Although the precursor lesion is an adenomatous polyp, patients do not have extensive polyposis as observed in FAP.

Lynch syndrome arises from a mutation affecting one of the MMR proteins responsible for repairing DNA replication errors, resulting in tumours arising through the MSI pathway as previously described. These tumours exhibit many of the phenotypical characteristics associated with the MSI pathway. Furthermore, tumours show a rapid progression through the process of carcinogenesis, and may be responsible for many 'missed' or interval cancers (26).

International criteria have been established to identify Lynch syndrome patients and families (Table 1.1). The Amsterdam criteria, first described in 1990, based likelihood of being a Lynch syndrome family on family history of colorectal cancer (86). Subsequent updates of these criteria included other non-colorectal cancer cancers associated with Lynch syndrome, with increased sensitivity for the detection of affected families (87). The Bethesda guidelines were subsequently established to identify colorectal cancers that should be tested for MSI, taking into consideration histopathological characteristics associated with MSI tumours (88). Not all tumours fitting these guidelines will exhibit evidence of MMR deficiency. Such tumours may fall into a separate category harbouring an as yet unknown mutation, and are termed Familial Colorectal Cancer Type X (28).

Table 1.1 Criteria for the diagnosis of Hereditary Non-Polyposis Colorectal Cancer

Amsterdam II criteria (87)
At least three relatives with hereditary HNPCC-associated cancer (tumours should be verified histologically whenever possible): 1. One is a first-degree relative of the other two; 2. At least two successive generations affected; **3.** At least one of the HNPCC-associated cancers diagnosed <50 years of age; **4.** Familial adenomatous polyposis has been excluded in colorectal cancer cases.
Bethesda Guidelines (88)
1. Colorectal cancer diagnosed in a patient <50 years of age; 2. Presence of synchronous/ metachronous colorectal or other HNPCC-associated tumours, regardless of age; 3. Colorectal cancer with MSI histology diagnosed in a patient <60 years of age; 4. Colorectal cancer or other HNPCC-associated tumour diagnosed in at least one first-degree relative <50 years of age; 5. Colorectal cancer or other HNPCC-associated tumour diagnosed in two first- or second-degree relatives at any age.

Hamartomatous polyposis syndromes

Hamartomas are benign overgrowths of cells arising from their tissues of origin. A number of hamartomatous polyposis syndromes have been described, including Peutz-Jeghers syndrome, juvenile polyposis syndrome and *PTEN* hamartoma tumour syndrome. In addition to a risk of colorectal cancer, these syndromes are commonly associated with extra-intestinal malignancies and manifestations. The risk of colorectal cancer varies; for example, there is a 34-fold increased risk in patients with juvenile polyposis syndrome compared to a greater than 500-fold increased relative risk of colorectal cancer in patients with Peutz-Jeghers syndrome (89). Overall, the hamartomatous polyposis syndromes

account for less than 1% of colorectal cancers. The various hamartomatous polyposis syndromes display an autosomal dominant inheritance pattern, with a number of different mutations identified in key tumour suppressor genes, such as *PTEN, SMAD4* and *LKB1* (90). The underlying mechanism leading to the development of an invasive cancer is not entirely clear. The presence of mesenchymal elements within associated polyps and cancers has led to the suggestion that mesenchymal overgrowth leads to 'landscaping' of the epithelium and carcinogenesis (91). However, given that germline mutations also exist in the epithelium, it is likely that carcinogenesis may occur due to loss of tumour suppressor function as described above.

1.3.1 Summary – aetiology of colorectal cancer

The aetiology of colorectal cancer is complex, incorporating genetic alterations, host characteristics and exposure to environmental factors. As colorectal cancer is sporadic in the majority (at least 80%) of cases, it is clear that the environment and host play an important role in carcinogenesis. Many of these factors co-exist as part of the 'Westernised lifestyle', hence the high incidence of colorectal cancer in developed countries and the increasing risk in developing countries adopting Western lifestyles.

The presence of a chronic systemic inflammatory response is also associated with colorectal cancer risk. Chronic inflammation links many of the host factors described above, including inflammatory bowel disease, obesity, the metabolic syndrome and atherosclerosis. Given this, it would suggest that a chronic inflammatory response might be a potential target for colorectal cancer prevention and treatment. The potential prophylactic effects of anti-inflammatory agents such as aspirin, NSAIDs and statins further ratify this. Indeed, such drugs are likely to have a role in the chemoprophylaxis of colorectal cancer, however whether they may have a role in its treatment following diagnosis remains to be established.

1.4 Management principles of colorectal cancer

1.4.1 Presentation

Patients with colorectal cancer may commonly present with rectal bleeding, alteration of bowel habit or an abdominal mass. The clinical presentation is often dictated by tumour location; whereas patients with colorectal cancer distal to the splenic flexure commonly present with rectal bleeding and alteration of bowel habit, patients with more proximally located tumours may present with an abdominal mass, iron deficiency anaemia or clinical evidence of intestinal obstruction. Typically patients present with a combination of symptoms rather than a single symptom (92). For example, patients presenting with rectal bleeding without alteration of bowel habit have a low risk of colorectal cancer (positive predictive value 2.5%), whereas those patients presenting with rectal bleeding and altered bowel habit but without any perianal symptoms have a much higher risk (positive predictive value 19.7%) (92). Stratification on the basis of age and symptom profile may aid in the identification of those patients who would benefit from fast-track referral from primary to secondary care and expedited investigations (Figure 1.2) (55, 93).

Up to one third of patients with colorectal cancer may present initially as an emergency (94), and often with locally advanced or distant metastatic disease. Even when controlling for patient and tumour-related factors, such as age and disease stage, emergency surgical resection confers poorer survival compared to patients undergoing elective resection, with an almost two-fold increased risk of cancer-associated mortality (94).

1.4.2 Population based screening

Population based screening of asymptomatic patients for colorectal cancer has been incrementally introduced across Scotland since 2007, with the rationale of reducing mortality by identifying patient with earlier stage colorectal cancer. On a biennial basis, individuals aged 50-74 are sent a guaiac-based faecal occult blood test and are

subsequently invited to attend for colonoscopy if the returned test is strongly positive (95). In patients with a weakly positive or spoilt test, a faecal immunochemical testing kit is sent; if positive, patients are again invited to attend for colonoscopy. Although the positive predictive value of guaiac-based testing is low, meta-analysis has shown a reduction in cancer mortality of 25% when adjusted for screening uptake (96). Furthermore, in addition to a decrease in the percentage of patients presenting as an emergency (97), a migration towards earlier stage disease has also been observed over subsequent rounds of screening (98).

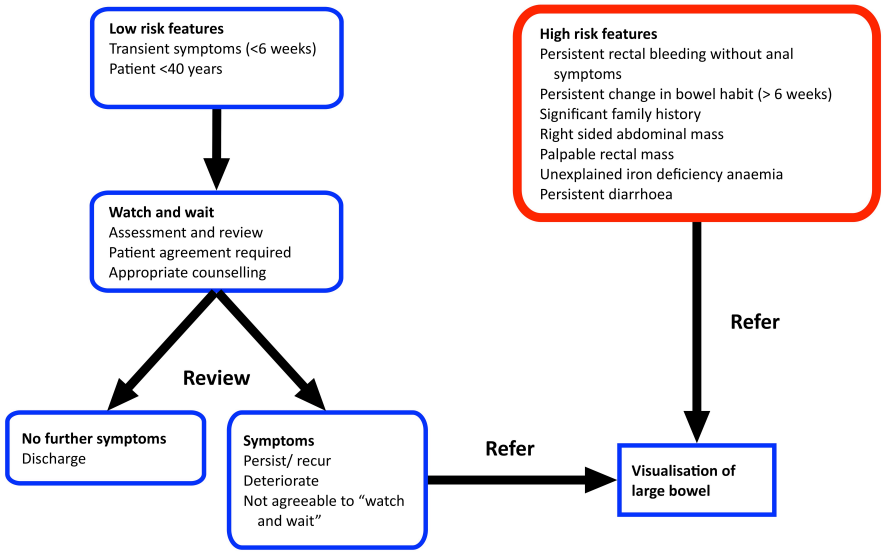


Figure 1.2 Guidelines for referral to secondary care for investigation of lower gastrointestinal symptoms. Adapted from SIGN guideline 126 (55)

1.4.3 Investigation

The investigation of patients with colorectal cancer aims to confirm a histological diagnosis. This may not be possible for all patients; for example, in the patient presenting as an emergency with peritonitis or intestinal obstruction, both histological diagnosis and

complete disease staging will often occur following emergency surgery. Likewise, although histological confirmation of colon cancer should ideally be performed prior to elective surgical resection, this may not always be feasible due to technical limitations of colonoscopy and biopsy technique. In such cases it would be appropriate to proceed to surgery in a patient with a highly suspicious colonic lesion and signs or symptoms suggestive of colon cancer. In patients with rectal cancer however, where neoadjuvant radiotherapy may be considered, or surgery may result in a permanent stoma or altered continence, histological confirmation of cancer is mandatory.

Flexible sigmoidoscopy and colonoscopy

Endoscopic assessment of the colon and rectum remains the gold standard for the diagnosis of colorectal cancer and should be offered to patients without major comorbidity (99). In addition to direct visualisation of the tumour, it allows for biopsy of lesions for histological diagnosis and, in the case of early stage polyp cancers and pre-malignant adenomata, may allow complete endoscopic resection. Direct visualisation of the distal colorectum from the anal verge to descending colon is possible at flexible sigmoidoscopy, and is likely to identify at least 70% of all cancers (100), without need for intravenous sedation or full mechanical bowel preparation. However confirmation of a cancer at sigmoidoscopy necessitates the need for full colonoscopic assessment of the colon, as synchronous tumours can occur in approximately 5% of cases (101). Colonoscopy requires full mechanical bowel preparation to allow for adequate visualisation of the colon, and patients often require intravenous sedation for comfort. To ensure adequate standards for colonoscopy, endoscopists should aim for caecal intubation in at least 90% of patients (101), however multiple factors, including inadequate bowel preparation, patient discomfort and experience of endoscopy practitioners may influence this. Both sigmoidoscopy and colonoscopy have an associated risk of perforation of around 0.1% (101).

Barium enema

Double-contrast barium enema examination is performed using rectal administration of barium and insufflation of air per rectum. Barium enema has previously been recommended in patients with major comorbidity deemed not fit for colonoscopy as well as in patients who have had an incomplete colonoscopy (99). The procedure however involves ionising radiation and requires complete bowel preparation and a similar level of patient mobility as would be expected during colonoscopy. Furthermore, identification of a lesion at barium enema may necessitate an attempt at colonoscopy to obtain histological diagnosis. Due to the presence of concomitant sigmoid diverticulosis in many patients, imaging of the distal colon and rectum is often suboptimal and additional flexible sigmoidoscopy is recommended to exclude a distal cancer (99). The procedure has largely been superseded by the introduction of computed tomographic (CT) colonography.

Computed tomographic colonography

CT colonography has a higher detection rate for colorectal cancer and is better tolerated by patients than barium enema (102, 103). Furthermore, in addition to assessment of the colon, it also allows for identification of extra-colonic pathology including metastatic disease. Similar to barium enema and colonoscopy, full mechanical bowel preparation is ideally required, however minimal preparation with administration of oral contrast only (faecal tagging) may be performed in patients unable to tolerate full bowel preparation with acceptable sensitivity and specificity (104, 105). Again, similar to barium enema, the procedure involves exposure to ionising radiation, and identification of a colonic lesion may require subsequent colonoscopy for histological diagnosis.

1.4.4 Pre-operative assessment of disease stage

Once a diagnosis of colorectal cancer has been histologically confirmed, further investigation is performed to stage the extent of disease both locally and with respect to

distant organ metastases. Staging guides both operative and non-operative management, particularly in patients with rectal cancer where accurate staging will not only determine surgical technique, but also the need for pre-operative chemotherapy and radiotherapy.

Computed tomography

Pre-operative CT of thorax, abdomen and pelvis should be offered to all patients to assess the presence of pulmonary, hepatic and other distant organ metastases (55, 99, 101). Intravenous iodinated contrast is administered unless contraindicated due to risk of sensitivity or contrast-induced renal failure. In such circumstances, a non-contrast enhanced CT can be performed, albeit with lower sensitivity. Although primarily indicated for staging of metastatic spread, pre-operative CT may also inform local disease staging with respect to T and N stage, particularly when multiplanar reformatting is utilised (106).

Magnetic resonance imaging

The use of magnetic resonance imaging (MRI) is superior to contrast-enhanced CT for local staging of patients with rectal cancer (55, 99, 101). Assessment of depth of tumour invasion, circumferential resection margin involvement, the presence of suspicious perirectal lymph nodes and extramural venous invasion using MRI can determine risk of local recurrence (Table 1.2), and aid in the selection of patients for primary resection or neoadjuvant chemoradiotherapy before surgery.

Pre-operative MRI in rectal cancer is of greatest value in determining depth of primary tumour growth and involvement of the circumferential resection margin (107, 108). Patients with tumour present within 1mm of the mesorectal fascia on MRI should be considered as high risk for circumferential resection margin involvement and should be considered for preoperative therapy. Furthermore, following neoadjuvant therapy, MRI assessment of tumour regression grade may be a useful prognostic marker prior to surgical resection (109).

Table 1.2 Rectal tumour characteristics as predicted by magnetic resonance imaging and risk of local recurrence. Adapted from NICE guidelines (99)

Local recurrence risk	Tumour characteristic predicted by MRI
High risk	• Tumour <1mm from or breaching circumferential resection margin, or • Tumour encroaching inter-sphincteric plane, or • Involvement of levator ani complex
Moderate risk	• Radiological cT3b or greater without threatened resection margin, or • Suspicious lymph node not threatening resection margin, or • Presence of extramural venous invasion
Low risk	• Radiological cT1, cT2 or cT3a, and • No lymph node involvement

Endoluminal ultrasound

Endoluminal ultrasound (EUS) may be considered for patients in whom MRI is contraindicated or in whom local excision for an early tumour is being considered (55, 99). Both T stage and N stage may be accurately determined (110, 111), however EUS may have greatest utility in identifying patients with carcinoma in situ who may be adequately treated by endoscopic excision only (112).

Positron emission tomography

Positron emission tomography-CT (PET-CT) combines functional assessment of abnormal tissue metabolism with anatomical detail derived from conventional CT imaging. In patients with colorectal cancer, 18-fluoro-deoxy-glucose is commonly utilised as a

radiotracer which provides a measure of glucose uptake and tumour metabolism. Positron emission tomography-CT may be utilised in patients being considered for curative resection of hepatic and pulmonary metastases to identify occult metastatic disease (55). In a case series of 102 patients being considered for potentially curative resection of metastatic colorectal cancer, the use of PET-CT avoided unnecessary laparotomy in 16 patients (113). In addition, PET-CT may also be of use in patients undergoing surveillance following colorectal cancer resection with a rising carcinoembryonic antigen (CEA) and normal CT imaging, or in those patients with possible radiological evidence of pelvic recurrence following treatment of rectal cancer (55).

Although the use of PET-CT is not routine in the staging of patients with colorectal cancer, colorectal neoplasms may occasionally be detected as an incidental finding on PET-CT performed in the staging of other cancers (55).

1.4.5 The multidisciplinary team

All patients with colorectal cancer should be discussed at the colorectal cancer MDT prior to the initiation of treatment, as well as following the completion of neoadjuvant therapy or surgical resection to determine the need for further treatment (101). As a minimum, the colorectal cancer multidisciplinary team (MDT) should comprise of at least two specialist colorectal surgeons, gastrointestinal clinical oncologists, histopathologists and diagnostic radiologists with gastrointestinal expertise, and colorectal clinical nurse specialists, in addition to ancillary clerical staff.

Implementation of the MDT process has led to improvements in the surgical and non-surgical management of colorectal cancer, with a subsequent improvement in survival (114, 115). For example, the management of locally advanced rectal cancer, with respect to increased use of neoadjuvant therapy and negative resections margins, has been shown to improve following introduction of routine MDT discussion (115). Furthermore, patients

with potentially resectable hepatic or pulmonary metastases may benefit from review by specialist MDTs comprising of thoracic and liver surgeons with expertise in metastatic disease (55). The development of standardised referral guidelines may aid in the identification of patients with metastatic disease likely to benefit from referral to such specialist MDTs (116).

1.4.6 Treatment of primary operable colorectal cancer

Surgery

Surgery remains the mainstay of curative treatment for colorectal cancer at present. The intent of curative surgery is twofold; resection of the tumour en bloc with a margin of healthy tissue to achieve macroscopically clear surgical margins, and resection of draining lymph nodes to remove potential lymph node metastases and allow proper pathological staging. Resection can be performed by open, laparoscopic and robotic techniques, and the operation performed depends on the anatomical location of the tumour, with resection margins following the vascular supply to the bowel to ensure an adequate oncological resection of the vascular pedicle and draining lymph nodes. Tumours within the caecum, ascending colon and hepatic flexure are resected by right hemicolectomy. Tumours of the transverse colon and splenic flexure are resected by an extended right hemicolectomy which is perceived as a safer procedure compared to limited segmental resection (101). Left hemicolectomy and sigmoid colectomy are performed for tumours arising in the left and sigmoid colon respectively.

Surgery for cancers arising within the lower two thirds of the rectum are resected either by anterior resection or abdominoperineal resection (APER), both of which have been revolutionised by the concept of total mesorectal excision (TME) (117). The TME approach follows the plane encompassing the mesorectum below the peritoneal reflection; sharp dissection along the mesorectal plane under direct visions preserves the hypogastric

plexus, ensuring pelvic nerve function is maintained, whilst reducing the risk of involved margins and local recurrence.

Comparing across trials, the introduction of this standardised surgical approach alone was shown to have similar effects on local recurrence rates as the introduction of neoadjuvant radiotherapy (118). Numerous trials have examined the role of TME compared to standard resection of rectal cancer (119-121). Such studies have benefited from standardisation of surgical technique, ensuring that involved surgeons adhere to the principles of TME. Similarly, a standardised approach to the reporting of rectal cancer resection specimens was also adopted. However, despite employing such methodological rigour, TME trials did not initially report on measures of resection quality, such as circumferential resection margin or excision plane, and their subsequent effect on survival. Indeed, retrospective reporting of the Dutch TME trial found an incomplete mesorectal excision had been performed in approximately one quarter of patients, with an associated increased risk of recurrence (122). However, despite not controlling for such factors, TME trials overall have reported superior oncological outcome with this approach, and it is now accepted as a standard of care in the surgical management of rectal cancer.

In patients with low rectal cancer, where the anal canal or levator ani muscle complex may be compromised, APER is required. Although traditionally associated with a relatively high rate of circumferential margin involvement, adoption of a more radical, extralevator approach, whereby the levator muscles are resected with the surgical specimen, is associated with a lower rate of circumferential margin involvement (123-125).

Minimally invasive surgery

The past two decades have seen significant advances in the operative and peri-operative management of patients undergoing resection of colorectal cancer. In particular, the

adoption of minimally invasive techniques, such as laparoscopic resection, has led to significant improvements in short-term outcomes of patients undergoing surgery.

Although potentially associated with an increased length of operation time, laparoscopic colonic resection has been shown to be associated with several short-term benefits; in addition to improved cosmesis from smaller wounds, the laparoscopic approach is associated with reduced post-operative analgesic requirements, reduced time to return of gastrointestinal function, and shorter time until discharge (126-128). Despite this, initial reports doubted the oncological benefits of laparoscopic versus conventional open resection, particularly with respect to achievement of negative resection margins and a potentially increased risk of laparoscopic port site metastases (129). However, several non-inferiority randomised controlled trials comparing laparoscopic to open resection of colon cancer have suggested oncological equivalence between the two techniques. The COSTS trial found laparoscopic resection not inferior to open resection for the primary outcome of disease recurrence (130). Although the COLOR trial could not exclude a difference in three-year survival favouring open resection, any difference was felt clinically insignificant and likely outweighed by the recognised short-term benefits of laparoscopy (131). Finally, the MRC CLASICC trial examined the clinical endpoint of resection margin positivity as an indicator of risk of local recurrence (128). Both approaches were equivalent with regards to resection of colon cancer, however laparoscopy appeared to be inferior to open resection for circumferential margin positivity in patients undergoing anterior resection for rectal cancer; long-term survival was equivalent for patients with colon cancer (132). Taken together, these results advocate the use of laparoscopic resection for patients with colon cancer, predominantly due to significant improvements in short-term outcomes and likely no difference in long-term survival.

The benefit of laparoscopic resection for patients with rectal cancer is not entirely clear. Although possibly suggesting a higher rate of circumferential margin involvement, long-term follow-up of CLASICC suggested no difference in survival with open or laparoscopic rectal cancer resection, albeit this was without the standardised use of extralevator abdominoperineal resection and with relatively low rates of neoadjuvant therapy (132). Similarly, the COLOR II and COREAN trials found no significant differences in locoregional recurrence or survival (133, 134). Conversely, the ALaCaRT and ACOSOG Z6051 randomised trials suggested a higher risk of margin positivity in patients undergoing laparoscopic resection (135, 136); however, although unable to recommend laparoscopic resection based on these pathological parameters, the effect on local recurrence and long-term survival in these two studies remains to be determined.

In addition to the advent of laparoscopic resection, the use of other minimally invasive techniques may significantly change the operative management of patients with colorectal cancer. Robotic-assisted surgery may improve the outcome of rectal cancer resection, particularly with respect to preservation of pelvic nerve function (137). However, both the oncological and cost benefit of robotic-assisted surgery remains to be determined.

Similarly, local excision may be indicated for some early tumours (55). This may be performed by either transanal endoscopic microsurgery for rectal cancers, or by polypectomy or endoscopic mucosal resection during colonoscopy for colon cancers. Generally, local excision is reserved for patients with T1 tumours and low risk characteristics on pathological assessment. Evidence of extensive submucosal invasion, tumour present within 1mm of the resection margin, lymphovascular invasion or poorly differentiated tumour would be indications for further surgical resection, as each of these increases the risk of lymph node involvement. The decision between local excision and surgical resection must be carefully considered with the patient, ensuring they are adequately informed regarding the risks of surgical morbidity versus the risks of

recurrence. In patients with a high risk of surgical morbidity, local excision, even with the presence of high-risk pathological characteristics, may be the preferred option.

Neoadjuvant radiotherapy

Patients with rectal cancer have a relatively higher risk of local recurrence given the natural confines of the pelvis. The addition of radiotherapy (either pre-operatively or post-operatively) reduces the risk of local recurrence, however pre-operative radiotherapy has been shown to be superior to post-operative therapy (138). Despite this, in patients with an involved surgical margin who did not receive pre-operative radiotherapy, post-operative chemoradiotherapy remains an acceptable salvage therapy. Pre-operative radiotherapy may either be used as short course pre-operative radiotherapy (SCPRT) or as long course radiotherapy in combination with 5-fluorouracil-based chemotherapy. Patients with moderate risk of local recurrence but without mesorectal fascial involvement on MRI may benefit from SCPRT in the week immediately prior to surgery; even with the introduction of total mesorectal excision, the addition of SCPRT reduces the risk of local recurrence at five years from 11.4% to 5.8% compared to surgery alone (139). In patients with locally advanced disease at risk of mesorectal fascial involvement, chemoradiotherapy is utilised to downstage and shrink the tumour so that clear resection margins can be obtained. Chemoradiotherapy is given over a five-week period and then followed by an interval before proceeding to surgery. Such a regime has been shown to be superior to post-operative chemoradiotherapy in reducing local recurrence rates (140). The addition of chemotherapy to pre-operative radiotherapy further reduces risk of local recurrence and increases likelihood of tumour regression and complete pathological response (141). In patients with rectal cancer at low risk of local recurrence, radiotherapy may be avoided to avoid the potential toxic effects of radiotherapy.

Adjuvant chemotherapy

In addition to improvements in pre-operative staging and surgical technique, refinement of chemotherapeutic drugs and treatment regimes has led to an increase in survival of patients with colorectal cancer. Adjuvant chemotherapy is now a standard care for the management of patients with stage III (node positive) colorectal cancer. Factors that may influence the decision to proceed to adjuvant chemotherapy however, include patient's age, comorbidity status and patient preference. Standard regimes consisting of a thymidylate synthase inhibitor, 5-fluorouracil (5-FU), given in combination with leucovorin, a folic acid derivative, for approximately six months have been shown to increase overall, disease-free and recurrence-free survival compared to surgery alone (142-144). Oral, pro-drug equivalents of 5-FU, such as capecitabine, are now available, with similar long-term survival benefit as 5-FU but with less toxicity (145). Combination regimes, with the addition of oxaliplatin, are superior to 5-FU/leucovorin alone (three-year disease-free survival 78.2% versus 72.9%), however carry an increased risk of treatment-associated toxicity (146, 147).

The survival benefit conferred by adjuvant chemotherapy to patients with stage II (node negative) colorectal cancer is less clear, with randomised clinical trials showing little or no improvement in survival (148, 149). For example, the QUASAR-1 trial, incorporating almost 3000 patients with stage II disease, found a modest, 3.6% absolute improvement in overall survival at five-years with the use of adjuvant chemotherapy (149). As such, current guidelines do not recommend the routine administration of adjuvant chemotherapy for patients with stage II disease (55, 99, 150), instead recommending its use in patients with the presence of high-risk pathological characteristics, such as tumour perforation or venous invasion. Increasingly, molecular characteristics, such as the absence of MSI, may be used as predictive biomarkers to identify patients with stage II disease likely to gain benefit from adjuvant therapy (151).

1.5 Determining prognosis of patients with colorectal cancer

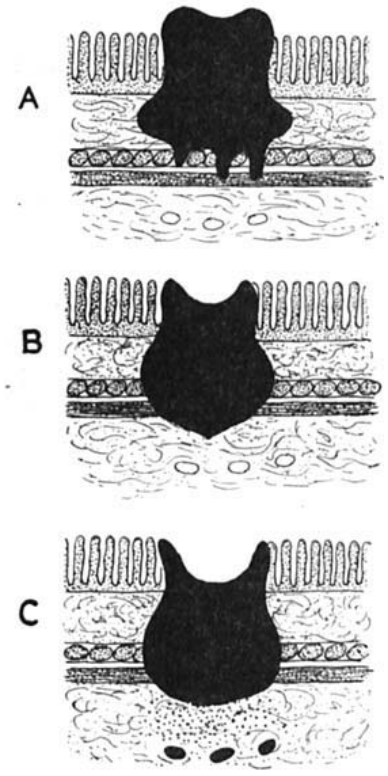
Overall, survival following potentially curative resection remains relatively poor, with approximately 50% of patients suffering disease recurrence within five years of surgery (152, 153). Pathological assessment of tumour characteristics is vital in determining prognosis and identifying patients likely to benefit from adjuvant therapy. For example, it is now accepted that adjuvant chemotherapy may benefit those patients with lymphatic metastases, whereas adjuvant radiotherapy may be of use in patients with positive circumferential resection margins following rectal cancer resection. Increasingly, other factors pertaining to the tumour, such as molecular characterisation and assessment of the tumour microenvironment, are recognised as having prognostic value in patients with colorectal cancer. In addition, host characteristics, such as age, emergency presentation, burden of comorbidity and the presence of local and systemic inflammatory responses are now recognised as important predictors of survival.

1.5.1 Tumour staging and prognosis of patients with colorectal cancer

Dukes' Stage

Cuthbert Dukes originally described a standardised means of staging rectal cancer in 1932 (154), which has since been validated for use in colon cancer. Dukes' staging is based on the previously held belief that lymphatic spread was not possible, or at least highly unlikely, in the absence of direct invasion though the bowel wall into extramural tissue. Using this staging system (Figure 1.3), tumours are classified as those without tumour growth through the bowel wall and without lymph node involvement (A), those extending through the bowel wall into surrounding tissues and without lymph node involvement (B), and those with lymph node metastases irrespective of extent of primary tumour growth (C). Three-year survival rates using this staging system were 80%, 73% and 7% for Dukes' A, B and C rectal cancer respectively (154). Since its original description, Dukes' staging has been modified several times. For example, Turnbull described a Dukes' stage D in 1967 to

denote the presence of distant metastatic disease (155). Presently, the Dukes and Bussey modification is recommended for routine pathological reporting by the Royal College of Pathologists (156). This modification further subdivides patients with stage C disease into those with involved lymph nodes but sparing of the highest or apical node (C1), and those with involvement of the apical node (C2).



Dukes' A Growth limited to wall of rectum

Dukes' B Extension of growth to extra-rectal tissues but no metastases in regional lymph nodes

Dukes' C Metastases in regional lymph nodes

Figure 1.3 Dukes' staging of rectal cancer. Adapted from Dukes' original description (154)

TNM staging

Although similar to the Dukes' staging system, the tumour, nodes and metastases (TNM) system has largely replaced its use in clinical practice and research. This more standardised staging system has allowed for more precise assessment of disease stage, with a view to better informing prognosis and need for adjuvant therapy. Universal adoption of TNM staging allows for more meaningful dissemination of information in the literature.

Denoix initially described the TNM system in 1946 (157), with the publication of the first edition of the "TNM pocket book" in 1968 by the Union for International Cancer Control (UICC). The American Joint Committee on Cancer (AJCC) also published TNM-based staging, however this differed from that of the UICC until 1987, when a unified staging system was proposed (158). The TNM system describes the size and depth of invasion of the primary tumour into or through the bowel wall (T stage), the degree of involvement of regional lymph nodes (N stage), and the presence of distant metastatic disease (M stage). Pre-operative imaging may allow for assessment of TNM stage prior to surgery, particularly with respect to the presence of distant metastatic disease. Although prognosis and need for adjuvant therapy will be determined by pathological assessment following surgical resection (pTNM stage), assessment of M stage is generally on the basis of radiological imaging rather than assessment of the resected specimen.

The TNM staging system is regularly revised to incorporate new evidence, with the 7th edition published in 2009 (159). This has, however, led to difficulties with respect to recruitment to and comparison of clinical trials due to differing criteria for each disease stage and subsequent stage migration over the recruitment period of clinical trials (160). Furthermore, recent revisions have been met with criticism due to a perceived lack of evidence with respect to many such changes (160). Presently, in the United Kingdom, the 5th edition of the TNM staging system (Table 1.3), published in 1997, is recommended by the Royal College of Pathologists for the routine reporting of colorectal cancer (101).

Whereas the Dukes' staging system was predicated on the hypothesis that lymphatic spread is uncommon without primary tumour growth through the bowel wall (154), the TNM classification accepts that lymphatic spread may occur even in the presence of an early primary tumour. Taking this into consideration however, both staging systems are broadly comparable. A comparison of Dukes' staging, TNM staging and associated five-year survival rates is displayed in Table 1.4.

It is clear that good surgical technique is required not only to ensure an adequate oncological resection is performed, but also to allow for accurate pathological staging of the patient (161). For example, a low yield of lymph nodes examined may result in under-staging of patients, and it is recommended that a minimum of 12 lymph nodes are examined to allow for accurate staging (101). Similarly, pathological assessment should follow rigorous protocols to ensure that resected specimens are examined and reported accurately and using reproducible methodologies. In keeping with this, the Royal College of Pathologists have recommended a minimum dataset with respect to reporting of colorectal cancer and have set minimum audit standards for the detection of key tumour characteristics, such as venous invasion.

1.5.2 Pathological characteristics and prognosis of patients with colorectal cancer

Although both Dukes' and TNM systems provide useful staging systems, it is increasingly appreciated that there is significant variation in prognosis within each staging group. Furthermore, an incremental increase in TNM stage does not necessarily translate into increased risk of recurrence, with some patients with stage II disease having a poorer outcome than those with stage III disease (162). Therefore, other pathological characteristics, such as tumour differentiation, venous invasion, and margin involvement are routinely assessed to aid in further stratification of patients. Such measures have been of greatest prognostic value in the assessment of patients with stage II disease, and may identify patients likely to benefit from adjuvant therapy (163).

Table 1.3 Summary of the 5th edition of the TNM classification of colorectal cancer

pT	**Primary tumour**
pTx	Primary tumour cannot be assessed
pT0	No evidence of primary tumour
pT1	Tumour invades submucosa
pT2	Tumour invades muscularis propria
pT3	Tumour invades into subserosa or non-peritonealised/ perirectal tissues
pT4	Tumour directly invades other organs or structures (pT4a) and/or perforates visceral peritoneum (pT4b)
pN	**Regional lymph nodes**
pNx	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastatic disease
pN1	1-3 regional lymph nodes contain metastatic disease
pN2	4 or more regional lymph nodes contain metastatic disease
pM	**Distant metastatic disease**
pMx	Distant metastatic disease cannot be assessed
pM0	No distant metastatic disease
pM1	Distant metastatic disease

Table 1.4 A comparison between Dukes' stage, TNM stage and five-year relative survival. Adapted from National Cancer Intelligence Network (164)

Dukes' stage	TNM stage	TNM classification	5-year relative survival
A	Stage I	T_{1-2}, N_0, M_0	93.2%
B	Stage II	T_{3-4}, N_0, M_0	77.0%
C	Stage III	Any T, N_{1-2}, M_0	47.7%
D	Stage IV	Any T, Any N, M_1	6.6%

Histological type

The majority of colorectal cancers are adenocarcinomas, however other rare tumour types, such as medullary, primary squamous and neuroendocrine, have been reported. Adenocarcinomas may exhibit features suggestive of mucinous (>50% of tumour composed of extracellular mucin) or signet ring (>50% of tumour displaying signet ring cell morphology) differentiation. Signet ring cell differentiation, in particular, is a stage-independent predictor of poorer survival (165). Mucin production may be induced by pre-operative chemoradiotherapy, and as such its prognostic value is less clear (166, 167).

Tumour differentiation

Differentiation is based on microscopic assessment of the degree of gland formation and architecture within the tumour. Tumours are graded as either well, moderately or poorly differentiated. Poor tumour differentiation is identified by irregularly folded, distorted glands, or complete absence of gland formation. Furthermore, tumours may be undifferentiated, bearing no resemblance to the underlying tissue architecture. Tumours are often heterogeneous in appearance, and for that reason differentiation grade is based on the predominant pattern within the tumour (168). Assessment of differentiation is subjective and may be prone to significant inter-observer variability (169). As such, in

clinical practice moderately- and well-differentiated tumours are often categorised together as low grade whereas poorly-differentiated and undifferentiated tumours are categorised as high grade (168). High grade/ poor differentiation is associated with poorer survival (170).

Peritoneal involvement

Peritoneal or serosal involvement occurs when tumour breaches the visceral peritoneum with evidence of tumour cells on the peritoneal surface or present within the peritoneal cavity. Using the 5th edition of TNM staging, this is defined as T4b disease. Direct continuity with the primary tumour is evident in the presence of peritoneal involvement, and this must be differentiated from peritoneal metastatic deposits occurring separately from the primary tumour which are indicative of metastatic disease. Peritoneal involvement is associated with an increased risk of intraperitoneal recurrence and reduced survival (171, 172).

Tumour perforation

A macroscopically visible defect through the tumour resulting in communication with the lumen is considered a tumour perforation, and is defined as T4b disease in the 5th edition of TNM. Although perforation may occur spontaneously in T4 tumours, it may also be iatrogenic, particularly in rectal cancers (173). The presence of tumour perforation is an adverse prognostic factor associated with increased risk of local recurrence and reduced survival (172, 173).

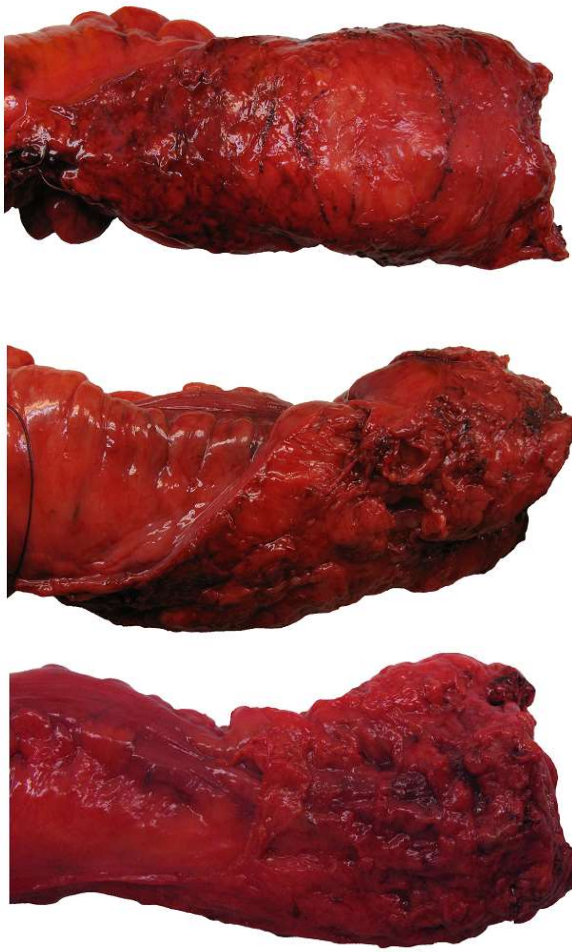
Resection margins

Resection margins include the resected ends of the specimen (longitudinal margins) as well as the circumferential resection margin. In specimens where the primary tumour is at least 30mm from either end of the specimen, longitudinal margins are not generally assessed for disease involvement. Involvement of the circumferential margin is an important

prognostic factor in patients with rectal cancer, associated with an increased risk of local recurrence (174, 175). More recently however, it is recognised as an adverse characteristic in cancers of the colon (172). Tumours that are completely excised are classified as R0, whereas those with microscopic involvement (≤1mm from resection margin) are classified as R1, and those with macroscopic, visible tumour at the margin classified as R2 (176). In addition to the presence of the primary tumour, the presence of tumour within lymphovascular channels and lymph nodes, as well as metastatic deposits in close proximity to the resection margin are also considered as evidence of an involved margin.

Plane of excision and specimen quality

Assessment of the plane of excision in patients undergoing TME surgery for rectal cancer is an important prognostic factor, with the risk of recurrence lowest in those patients with a complete mesorectal excision (122). The plane of excision may be graded (Figure 1.4), with an increasing risk of local recurrence observed with decreasing quality of resection (177). Similarly, performing a resection out with of the plane of the levator sling during APER (extralevator approach) reduces the risk of "waisting" of the specimen and risk of an involved resection margin (124). Although the plane of resection may be influenced by several tumour and patient factors, such as depth of invasion, sex and body habitus, it is also a useful metric for quality of surgery (122, 178). As such, plane of surgical resection should be routinely reported for patients undergoing resection of rectal cancer and discussed at MDT to aid in the improvement of surgical quality.



Mesorectal plane of excision

Specimen exhibits shiny fascial covering with no defects and a good bulk to mesorectum anteriorly and posteriorly.

Intramesorectal plane of excision

Specimen exhibits minor irregularities in mesorectum with defects which do not extend into the muscularis propria.

Muscularis propria plane of excision

Specimen exhibits significant defects in mesorectum which extend to muscularis propria.

Figure 1.4 Examples of planes of excision observed in anterior resection for rectal cancer. Adapted from Loughery et al. (179)

Number of lymph nodes examined and lymph node ratio

An increased number of lymph nodes examined following surgery is associated with improved survival of patients with node negative (stage I/II) colorectal cancer (180). This may reflect more accurate staging, with a low lymph node yield effectively under-staging the patient. For this reason, the Royal College of Pathologists recommend that a minimum of 12 lymph nodes are examined (101). Similarly, the American Society of Clinical Oncology (ASCO) consider a low lymph node yield (≤10) as an indication for adjuvant therapy in patients with stage II disease (150).

In patients with stage III colorectal cancer, an increased number of lymph nodes examined is also associated with increased survival (180). Furthermore, assessment of the ratio of number of positive lymph nodes compared to total number examined, or lymph node ratio (LNR), has been shown to be an independent prognostic characteristic, with an increasing LNR associated with poorer survival (181). Inclusion of LNR has been suggested as a superior marker of lymph node metastases than pN stage (182). However, several thresholds have been reported in the literature (182-185), with a lack of validation precluding the routine use of the LNR in clinical practice.

Venous invasion

Venous invasion is defined by the presence of tumour cells within an endothelial-lined space which either contains red blood cells or is surrounded by a rim of muscle (186). The 'orphan artery' sign, where an elongated tumour deposit is identified adjacent to an artery is also indicative of venous invasion. The presence of venous invasion has been reported as a predictor of increased risk of systemic metastases and decreased survival in patients with rectal cancer (187), and has subsequently been validated as an independent prognostic marker in patients with colon cancer (172, 188, 189). Although early reports suggested that only extramural venous was a prognostic factor, more recent studies have suggested that intramural venous invasion may also be associated with survival (172, 188, 189). Detection rates for venous invasion have been shown to vary between institutions (190), and are influenced by the number of blocks and sections examined and techniques used (191). For example, in a single institution, the use of elastica staining in addition to haematoxylin and eosin (H&E) staining was shown to increase venous invasion detection rates from 18% to 58% (Figure 1.5), with a resultant increase in prognostic value (188).

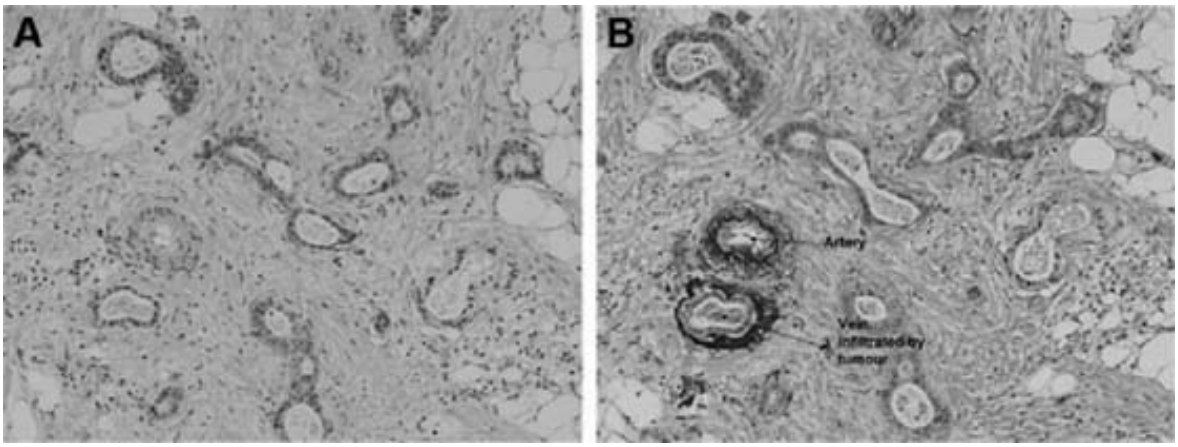


Figure 1.5 Extramural venous invasion as evidenced by haemotoxylin & eosin **(a)** and haemotoxylin & eosin and elastic staining **(b)**. Figure from Roxburgh, McMillan et al. (188)

Perineural invasion

Perineural invasion is characterised by tumour invasion of nerve structures with spread along the nerve sheath (192). The presence of perineural invasion is indicative of an aggressive tumour phenotype, and is an adverse prognostic characteristic associated with increased risk of locoregional recurrence and decreased survival (193-195). Furthermore, the presence of perineural invasion is an independent predictor of lymph node metastases, and may be an indication for more radical surgery following local excision of T1/T2 tumours (196). Assessment of perineural invasion is not presently recommended by the Royal College of Pathologists as part of the minimum dataset for colorectal cancer reporting and is likely to be underreported in routine clinical practice. In a retrospective re-review of 269 patients with colorectal cancer, perineural invasion was identified in 22% of patients compared to 0.5% in the original prospective reports (197).

Determining prognosis of patients with Stage II colorectal cancer

Whereas adjuvant chemotherapy is generally recommended for patients with stage III colorectal cancer, identifying patients with stage II disease who may benefit is more problematic. In 2002, Petersen and colleagues described a scoring system based on the

presence of high-risk pathological characteristics to aid in the identification of patients with Dukes' B/ stage II colon cancer at increased risk of recurrence (172). Using this prognostic index, patients were allocated one point each for the presence of venous invasion, margin involvement and peritoneal involvement, and two points for the presence of tumour perforation. A cumulative increase in score was associated with poorer survival (Table 1.5), and the authors recommended a cut-off of 2 or more to indicate high-risk, node negative disease. Indeed, the survival of patients with low-risk disease is comparable to that of patients with Dukes' A disease, whereas patients with a high-risk prognostic index disease have poorer survival than those with single node positive Dukes' C colorectal cancer (163).

Table 1.5 The relationship between Petersen Prognostic Index and five-year cancer specific survival of patients with Dukes' B colon cancer. Adapted from (172)

Prognostic Index	**Total patients**	**5 year cancer-specific survival (95% CI)**
0	82	94.2% (85.0-97.8)
1	109	79.5% (69.9-86.3)
2	63	54.3% (40.3-66.3)
≥3	14	30.4% (7.8-57.4)
All	268	76.1% (70.0-81.0)

Similar guidelines for the identification of high-risk stage II disease have been published by ASCO (150). Using these guidelines, low lymph node yield (less than 10 nodes), T4 stage, tumour perforation or poor tumour differentiation are considered high-risk pathological characteristics which may be used to identify patients who may benefit from adjuvant therapy.

1.5.3 Molecular characteristics and prognosis of patients with colorectal cancer

In addition to histopathological characterisation of the tumour, it is increasingly appreciated that molecular characterisation of the tumour may aid not only in determining prognosis, but also in predicting response to therapy. Furthermore, assessment of tumour-related proteins, such as carcinoembyronic antigen (CEA), may be useful adjuncts in the follow-up of patients following potentially curative resection.

Microsatellite instability

Approximately 15% of colorectal cancers arise through the MSI pathway, with both prognostic and therapeutic implications. Two large meta-analyses, incorporating 32 and 31 studies respectively (151, 198), each found that MSI was associated with an improvement in overall survival of around 40% compared to MSS tumours. This survival benefit appeared to be greatest in patients with stage II/III disease. Of interest however, MSI status appears to confer a poor response to standard, 5-FU-based chemotherapy regimens (199), potentially due to the increased tolerance of tumour cells lacking functional mismatch repair proteins to the cytotoxic, DNA-damaging effect of these agents (16).

The favourable prognosis of patients with MSI colorectal cancer may reflect the decreased preponderance for MSI tumours to metastasise. It has however also been suggested that the rapid mutational rate associated with loss of MMR protein function leads to the development of multiple neo-antigens which may provoke a co-ordinated anti-tumour immune response as evidenced by the increased density of tumour-infiltrating lymphocytes within the tumour microenvironment. It is not entirely clear if the favourable prognosis associated with the MSI pathway is independent of the local inflammatory cell infiltrate (200, 201).

Oncogene and tumour suppressor gene expression

Given the importance of oncogene and tumour suppressor gene expression in the development of colorectal cancer, there exists a sound biological rationale to study the prognostic and predictive value of activating and de-activating mutations. However, few of these have shown promise as candidate genes capable of informing prognosis and treatment decisions. Many studies have failed to adopt standardised methodologies for the detections of mutations, such as is the case with *p53* mutations (202). Furthermore, the high frequency of some mutations in patients with colorectal cancer, such as *APC* mutations, limits their prognostic utility (11).

A different approach has been the identification of gene expression profiles capable of predicting recurrence risk, particularly in the context of stage II disease (203). One such profile is ColoPrint, an 18-gene expression classifier which may effectively identify patients at high and low risk, with reported five-year relapse-free survival of 67% and 88% respectively (204). However, the practicalities of the assay employed, which requires fresh frozen tissue, in addition to high costs prohibits routine clinical use of such techniques.

The proto-oncogene *KRAS* has been one of only a few gene assays to translate to routine clinical practice. Activating mutations of *KRAS* have been associated with reduced survival of patients with metastatic colorectal cancer, and predict lack of therapeutic and radiological response to epidermal growth factor receptor (EGFR) inhibitors (205). The clinical utility of *KRAS* mutations in patients with non-metastatic disease is unclear however, with some studies showing an association with reduced survival (206), and others showing no prognostic value (207). Mutated *BRAF* and *PIK3CA* are also of increasing clinical value in determining patients with metastatic disease unlikely to benefit from EGFR inhibitors (205). Furthermore *PIK3CA* mutations may predict benefit from aspirin (208).

1.5.4 Determining prognosis of patients with colorectal cancer – summary

Although disease stage is primarily based on pathological assessment of the extent of disease spread, it is clear that other tumour characteristics may determine both disease stage and likely benefit from adjuvant therapy. Many of these characteristics are based on pathological assessment of the resected tumour, with several now incorporated into routine assessment. Others however, such as perineural invasion and LNR have yet to translate to clinical practice. Furthermore, although it is clear that molecular and genetic analysis may similarly aid treatment decisions, lack of standardisation and high costs remain barriers to routine implementation.

1.6 The local and systemic environment in patients with colorectal cancer

Rather than being a tumour cell autonomous process, sustained tumour growth and dissemination is reliant on the presence of a tumour-supporting environment. Host inflammatory responses, at both the local and systemic level, play an important role in disease progression, as do other components of the tumour microenvironment. Assessment of both the local and systemic environment may aid in determining prognosis of patients with colorectal cancer, and could potentially identify patients likely to benefit from adjuvant chemotherapy and novel therapies.

1.6.1 Inflammation in cancer

Cancer and inflammation have long been recognised as overlapping phenomena. In the 19th century, the pathologist Rudolf Virchow described the 'lymphoreticular infiltrate' identifiable within cancer tissues, recognising that this likely reflected the origin of cancer at sites of chronic inflammation (209). Further supporting the link between inflammation and cancer is evidence that targeting chronic inflammation reduces cancer risk, as is evident in patients with longstanding IBD. In addition, a number of anti-inflammatory agents, including aspirin and NSAIDs, have been associated with a reduction in the risk of cancer, including colorectal cancer (70).

The recognition that inflammation is complicit in carcinogenesis is such that it is now considered a hallmark of cancer, with both tumour-initiating and enabling characteristics (210). However, counterbalancing this is the host anti-tumour immune response, hindering continued tumour growth and dissemination. Most if not all tumours elicit a combination of both responses, with amelioration of anti-tumour immunity responsible for tumour progression (211).

The host inflammatory response

Immunity and inflammation is controlled by a number of cell types and is ultimately responsible for protecting the host from pathogenic agents and injury. At its most basic level, the inflammatory and immune response can be considered as comprising of innate (non-specific) and adaptive (acquired or specific) cellular immune responses, connected and communicating via non-cellular, humoural immune responses (212). In addition to being responsible for the detection and elimination of microbes, the host inflammatory response is also capable of detecting aberrant host cells, including cancer cells, through the detection of neo-antigens.

The innate immune response is primarily comprised of phagocytic and granulocytic cells such as neutrophils (the most abundant leukocyte type), macrophages, eosinophils, dendritic cells and natural killer cells amongst others (212). Trauma and pathogens activate these cell types through pattern recognition receptors, with release of inflammatory cytokines and chemokines eliciting an inflammatory response and recruiting further inflammatory cell types to the site of injury. Although generally self-limiting, the innate immune response may persist as a chronic inflammatory response, as is observed in the tumour microenvironment of many tumours (213).

Adaptive immunity may be triggered by innate immunity and recognises 'non-self' antigens on microbes and cancer cells to provoke an immune response (214). In addition to eliminating pathogens, this immune response results in an immunological memory so that subsequent exposure to the same pathogen mounts a rapid response. The main effector cells of the adaptive immune response are T-lymphocytes and B-lymphocytes, which are activated by antigen presentation by antigen presenting cells such as dendritic cells. B-lymphocytes elicit a humoral response with the release of immunoglobulins (antibodies) which bind to pathogens to enable their recognition by other immune cells. T-

lymphocytes orchestrate a cell-mediated response, with a number of different subsets recognised, including helper, cytotoxic, memory and regulatory T-lymphocytes.

The humoural immune response is comprised of soluble macromolecules and links cellular components of the innate and adaptive immune responses. Innate components, such as complement, opsonins and pentraxins, including CRP, can induce destruction of pathogenic cells through osmotic lysis and facilitating phagocytic cell activity. Adaptive components include antibodies; in addition to assisting in pathogen recognition, antibody binding facilitates opsonisation and phagocytosis of pathogenic cells.

It is increasingly recognised that subsets of T-lymphocyte populations, particularly helper T-lymphocytes, may be polarised to have vastly different functions with both pro-tumour and anti-tumour properties (215). Whereas it was previously hypothesised that host innate and adaptive responses were respectively pro- and anti-tumour, it is increasingly appreciated that tumour-associated inflammation is far more intricate (Table 1.6). Indeed, a complex cascade of both adaptive and innate immune cells, inflammatory cytokine mediators and intracellular transcription factors are involved. Furthermore, the plasticity of such components of the host immune response mean that it is not just immune cell type, but the inflammatory milieu in which it is found which ultimately determines the nature of the tumour-associated inflammatory response.

Inflammation as a tumour-initiating factor

Chronic inflammation predisposes to carcinogenesis by increasing the rate of mutations within precursor cells through a number of mechanisms (215). DNA damage may be precipitated by the release of reactive oxygen and nitrogen species from chronic inflammatory mediators, such as neutrophils and macrophages. Further genomic instability also arises as a result of epigenetic silencing of MMR genes or direct inactivation of MMR enzymes. Furthermore, pro-inflammatory cytokine signalling

Table 1.6 Anti-tumour and tumour-promoting roles of cellular components of the innate and adaptive immune response. Adapted from Grivennikov, et al. (215)

Cell Type	Anti-tumour activity	Tumour-promoting activity
Innate Immune Cell Population		
Neutrophils	Direct cytotoxicity, regulation of cytotoxic T-cells	Production of inflammatory cytokines, proteases, reactive oxygen/ nitrogen species
Macrophages	**M1 phenotype:**	**M2 phenotype:**
Myeloid-derived suppressor cells	Antigen presentation, anti-tumour cytokines (IL-12, IFN)	Immunosuppression, production of inflammatory cytokines, chemokines, growth and angiogenesis factors
Dendritic cells		
Natural killer cells	Direct cytotoxicity, production of cytotoxic cytokines	
Mast cells		Production of inflammatory cytokine, chemokines
Adaptive Immune Cell Population		
B-cells	Tumour-specific antibody production	Production of inflammatory cytokines, mast cell activation, immunosuppression
Cytotoxic T-cells ($CD8^+$)	Direct cytotoxicity, production of cytotoxic cytokines	
Helper T-cells ($CD4^+$)	**Th1 phenotype:**	**Th2 phenotype:**
	Regulation of cytotoxic T-cells, anti-tumour cytokines	Stimulation/ education of macrophages, production of inflammatory cytokines
	Th17 phenotype:	
	Regulation of cytotoxic T-cells	**Th17 phenotype:**
	Regulatory:	Production of inflammatory cytokines
	Regulation of inflammation	**Regulatory:**
		Immunosuppression, production of inflammatory cytokines

promotes cell growth and the development of a 'stem cell' phenotype in pre-malignant cells. An increase in mutational burden itself is pro-inflammatory, further promoting a local chronic inflammatory state and perpetuating genomic instability (Figure 1.6).

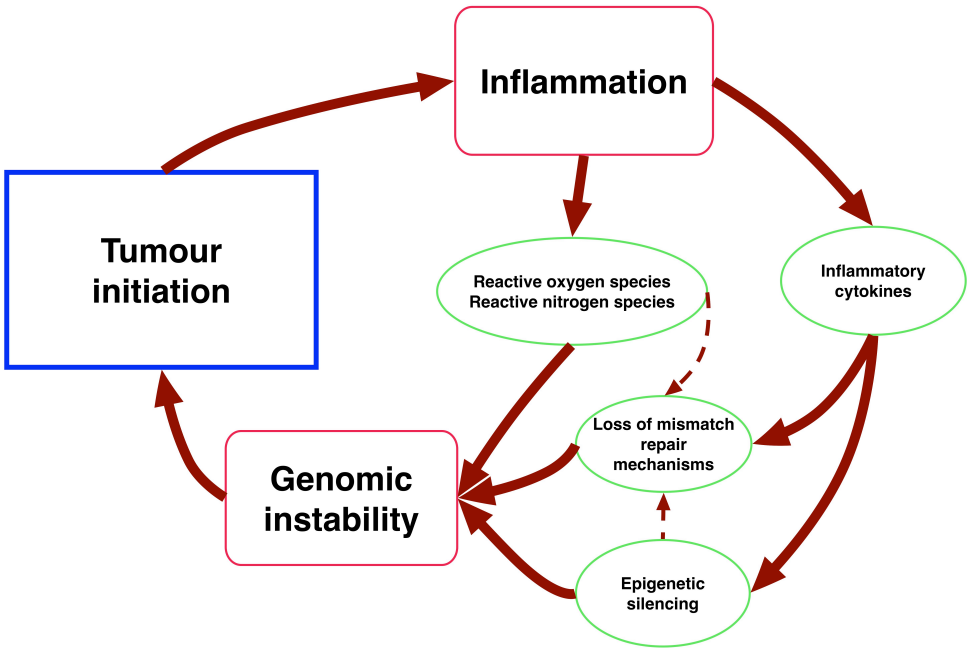


Figure 1.6 The relationship between tumour inflammation and genomic instability

Inflammation as a tumour-enabling factor

Chronic inflammation promotes tumour cell survival and eventual dissemination through a number of mechanisms. Activation of intracellular signal transduction pathways, such as Janus-activated kinase/ signal transduction and activator of transcription (JAK/STAT), nuclear factor kappa-B (NF-κB) and cyclo-oxygenase-2 (COX-2) have a multitude of pro-oncogenic effects, including increasing cell survival and proliferation and inhibition of apoptosis (211, 216, 217). In addition, activation of such pathways results in increased synthesis of pro-inflammatory cytokines, including interleukin-6 (IL-6) IL-17 and IL-22,

chemokines and growth-related factors. This cascade has a number of pro-tumour effects, including recruitment of inflammatory cells to the tumour microenvironment. Recruited neutrophils, M2-polarised macrophages and Th2-, Th17- and regulatory helper T-cells further promote ongoing tumour growth by release of inflammatory cytokines and prostaglandins, in addition to pro-angiogenic and growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-*B* (TGF*B*) (215). This inflammatory milieu down-regulates host anti-tumour immune responses, inhibiting effective tumour infiltration and destruction by cytotoxic T-cells and natural killer cells. In tandem with neo-angiogenesis and recruitment and transformation of resident fibroblasts to cancer-associated fibroblasts, this produces a tumour microenvironment supportive of tumour establishment and ongoing growth (218).

The inflammatory response also plays an integral role in the transition to disseminated disease and establishment of metastases. Activated inflammatory cells and secreted metalloproteinases and collagenases allow tumour cells to migrate through normal tissue barriers. The above processes may promote the loss of normal cell adhesion and epithelial-mesenchymal transitioning (EMT), a vital process required for tumour cells to 'break free' and disseminate (216). Furthermore, increased vascular permeability, which allows migration of inflammatory cells to the tumour microenvironment, may also allow tumour cells to migrate into the systemic vasculature. Finally, activated platelets aggregate around disseminated tumour cells, essentially 'shielding' them and facilitating the establishment of distant organ metastases (219).

Chemokines in cancer

Chemotactic cytokines, or chemokines, and their receptors play an important role in tumour survival, progression and dissemination. Chemokines are small peptides that mediate leukocyte migration (chemotaxis), however also facilitate normal and malignant

cell homeostasis (220, 221). Although chemokines represent a molecular superfamily with distinct structural similarities, they are classified into four subfamilies based on the location of the first two cysteine residues in their structure: CC, CXC, CX_3C and C (X represents other amino acid residues within their structure). Similarly, chemokine receptors are named on the basis of their chemokine ligands, although each ligand may activate numerous receptors, and each receptor may be activated by a number of ligands (220).

Chemokine and chemokine receptor expression within the tumour microenvironment is observed not only on cancer epithelial cells, but also on surrounding fibroblasts and immune and inflammatory cells (220). In addition to promoting leukocyte infiltration, activation of chemokine receptors by their respective ligands also induces signal transduction pathways with resultant effects on the cell cycle, proliferation, angiogenesis and cell adhesion. Similar to cellular components of the inflammatory response, individual chemokines may elicit both pro-tumoural and anti-tumoural responses depending on both the underlying cell type involved as well as the surrounding inflammatory milieu.

It is increasingly apparent that chemokine activity plays an integral role in not only facilitating EMT, but also in the development of distant organ metastases (221, 222). Indeed, rather than only shaping the tumour microenvironment, chemokine activity may also explain the tropism for metastatic cancer cells to specific organ sites. For example, CXCL12 expression in liver, lung and lymph nodes may promote migration of CXCR4-expressing colorectal cancer cells to these sites and initiation of metastases (222). Consistent with this, expression of CXCR4 in primary colorectal tumours is associated with increased risk of liver metastases and poorer survival (223). Given their multifaceted role in tumour biology, targeting of chemokines and chemokine receptors present one potentially attractive therapeutic option in patients with colorectal cancer (224).

Immune surveillance and cancer immunoediting

The recognition of tumour infiltrating lymphocytes as a favourable prognostic factor gives plausible evidence for the role of immune surveillance as an important host anti-tumour mechanism (225). However, it is clear that tumour cells may circumvent competent host immune responses through a number of mechanisms, recognised as the process of cancer immunoediting (226). This process is thought to comprise of three phases: elimination, equilibrium and escape. In the elimination phase, adaptive and innate immune responses readily recognise and destroy developing tumour cells, generating a T-cell-mediated response with immunological memory (227). However, as this process continues, selection pressure allows for the survival of cancer cells with reduced immunogenicity and capable of survival in an immunocompetent environment (immune equilibrium). As this progresses, host immune responses may be suppressed by a number of mechanisms, including down-regulation of tumour expression of major histocompatibility complex (MHC) class I expression, immune checkpoint activation and activation of immunosuppressive regulatory T-cells (211). In turn, immune escape allows for ongoing tumour growth and dissemination. Indeed, evidence of early metastatic spread in the absence of tumour infiltrating memory T-cells highlights the importance of tumour cells overcoming adaptive anti-tumour immunity to allow sustained growth (227).

Inflammation and signal transduction pathways in cancer

Intracellular signal transduction pathways control many facets of tumour cell growth and metabolism, in addition to influencing the host inflammatory response to cancer. Two of the most commonly studied pathways in the context of colorectal cancer are the JAK/STAT3 and NF-κB pathways. Both may be constitutively activated in neoplastic cells, more commonly through upregulation of upstream signalling rather than gain-of-function mutations (217). Although both STAT3 and NF-κB control normal physiological functions, activation in cancer cells leads to deregulation of several pro-tumour pathways

complicit in cell cycle progression, resistance against apoptosis and hypoxia, and angiogenesis. Likewise, tumour cell activation of STAT3 and NF-κB may impair host anti-tumour immunity.

Significant interaction exists between the STAT3 and NF-κB pathways. At the transcriptional level, there is overlap between the gene promoters which may be targeted by either pathway, and co-stimulation by both may result in even greater transcriptional activity of candidate genes (228). Furthermore, STAT3 and members of the NF-κB family may interact with each other; for example, STAT3 may induce NF-κB pathway activation, whereas members of the NF-κB family may inhibit STAT3 transcriptional activity (217, 218).

It is not surprising that NF-κB and STAT3 have been shown to play a pertinent role in both the initiation and progression of cancer. STAT3 is hypothesised to play a pivotal role in colitis-associated carcinogenesis (54), and tumour cell expression of STAT3 has been associated with poorer survival of patients with colorectal cancer. Of interest however, the role of both pathways with respect to initiation of carcinogenesis and manipulation of host inflammatory responses differs according to the cell type in which it is activated (Figure 1.7) (229). For example, whereas studies have shown that STAT3 ablation in normal colonic epithelial cells inhibits carcinogenesis (54), ablation in myeloid-derived cells enhances inflammation and carcinogenesis (230). Conversely, STAT3 inhibition in lymphocytes may enhance anti-tumour immune responses (231). Similarly, NF-κB shows differential activity on tumourogenesis and host immunity dependent on the studied cell type (217). Both STAT3 and NF-κB may be potential therapeutic targets with effects not only on tumour cell growth, but also host anti-tumour immune responses. However, given the plasticity of their effects on host immunity and tumour growth depending on the studied cell type, and the significant interaction between both pathways, further investigation of the effects of targeted inhibition is warranted.

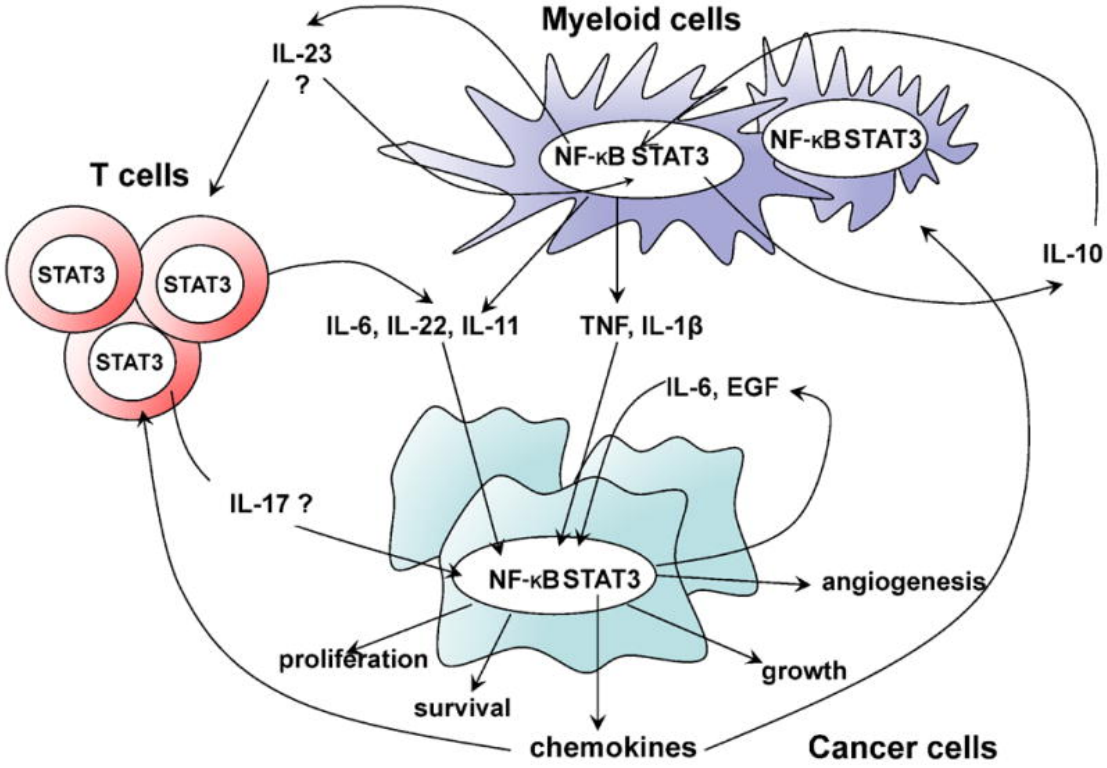


Figure 1.7 Tumour and immune cell STAT3 and NF-κB activation and their effects on tumour cell survival. Figure from Grivennikov and Karin (217)

Inflammation in cancer – summary

Inflammation plays an integral role in not only the elimination, but also the initiation and progression of cancer. Although host anti-tumour immune responses may identify and destroy nascent and established cancer cells, this may be circumvented by a number of mechanisms. Furthermore, as the tumour progresses, host inflammatory responses may be recruited to promote sustained tumour growth and dissemination. Underlying signal transduction pathways, such as STAT3 and NF-κB control numerous aspects of the host immune and inflammatory response to cancer, and ultimately may yield potential therapeutic targets.

1.6.2 The local inflammatory response and prognosis of patients with colorectal cancer

Given the above, it is not surprising that the local inflammatory infiltrate has been identified as a prognostic factor in patients with colorectal cancer. Since initial reports

over 80 years ago, a multitude of studies have reported improved survival in association with the presence of a conspicuous inflammatory cell infiltrate (225). Indeed, it is now clear that assessment of the inflammatory infiltrate may inform prognosis independent of TNM staging and molecular characterisation of colorectal cancer (201, 232).

Early reports primarily considered the presence of the generalised inflammatory cell infiltrate, encompassing both adaptive and innate immune cells, utilising H&E-stained specimens. As immunohistochemistry and molecular techniques have evolved, specific immune cell types, particularly T-lymphocyte subsets, and their location within the tumour microenvironment have been increasingly studied. However, it is of considerable interest that reports have shown these differing approaches strongly correlate; as the generalised peritumoural inflammatory cell infiltrate increases in density, lymphocyte density also increases, whereas neutrophil and macrophage density decreases (233).

Taken together, this would suggest that a conspicuous generalised inflammatory cell infiltrate signifies a co-ordinated anti-tumour response whereas an attenuated response represents a chronic, innate-driven pro-inflammatory response. However, whether crude assessment of the generalised inflammatory cell infiltrate offers similar prognostic value to more refined assessment of immune cell type, density and location remains to be determined. Furthermore, whether this information can be utilised to decide treatment is unclear. Indeed, it has been suggested that patients with a conspicuous inflammatory cell infiltrate, and as such a good prognosis, may be more likely to respond to 5-FU-based chemotherapy, whereas those patients with a poor response, and as such deemed high risk, may be less likely to gain benefit from adjuvant chemotherapy (234).

Despite reflecting the host anti-tumour immune response, several pathological characteristics may influence the density of the local inflammatory cell infiltrate, and as such may confound its clinical utility. For example, the presence of extensive tumour

necrosis has been associated with loss of the local inflammatory cell infiltrate in colorectal cancer (235). Similarly, stromal infiltration may result in ineffective immune cell infiltration of the tumour microenvironment (236, 237). Although not previously examined in the context of colorectal cancer, peri-tumoural oedema has previously been shown to correlate with the density of the local inflammatory cell infiltrate (238), and may also influence the local tumour microenvironment of patients with colorectal cancer. Whether such factors influence only density and not the nature of the local inflammatory cell infiltrate, remains to be fully determined. However, such factors may make assessment of the local inflammatory cell infiltrate more difficult, confounding its clinical utility.

Assessment of the generalised inflammatory cell infiltrate

Assessment of the generalised inflammatory cell infiltrate utilising H&E-stained specimens has shown considerable interest due to its reliance on routine specimens, and low associated costs. To date however, these measures have failed to translate into routine clinical practice due to a number of reasons, including lack of standardisation and validation as well as concerns regarding reproducibility.

Jass Criteria

One of the earliest and most recognised assessments of the tumour inflammatory cell infiltrate in colorectal cancer was described by the pathologist Jeremy Jass in the 1980s. Jass first published on the inflammatory cell infiltrate of rectal cancers as an independent prognostic factor in 1986, describing the presence of a pronounced lymphocytic infiltrate at the invasive margin of the tumour (239). Alongside this, he recognised the presence of a connective tissue lamina, resembling the lamina propria, which he hypothesised as a barrier to continued tumour growth. Subsequently, in 1987, assessment of the inflammatory cell infiltrate was incorporated into a five-point pathological scoring system

for rectal cancer which was able to stratify survival at five years from 94% to 27% (240). Using Jass criteria (Figure 1.8), alongside growth limited to the bowel wall, a low burden of lymph node metastases and an expanding invasive margin, the presence of a "distinctive and delicate connective tissue mantle or cap at the invasive margin of the growth in which lymphocytes and other inflammatory cells were scattered" is recognised as a good prognostic feature.

The Jass criteria has failed to translate into clinical practice, partly due to limited clinical utility when compared to other assessments of the local inflammatory cell infiltrate and even conventional Dukes' staging (241-243). Furthermore, difficulties with respect to reproducibility outside of specialist pathology units has limited its routine adoption (242, 244).

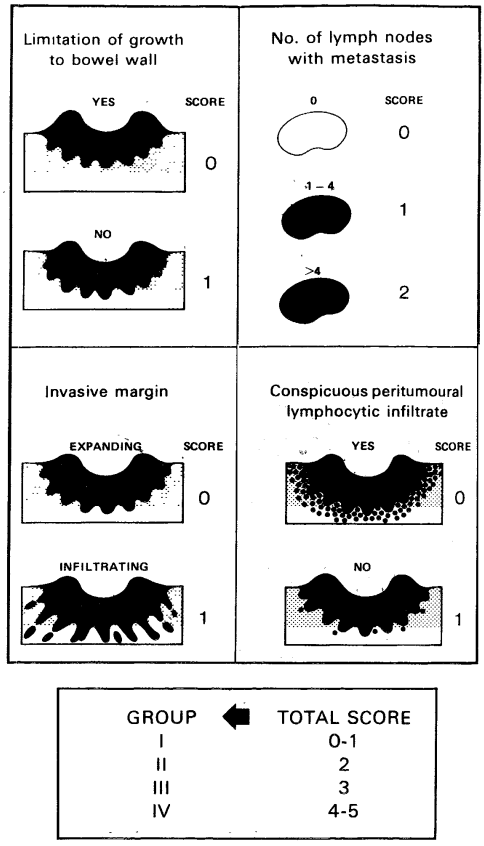


Figure 1.8 The Jass criteria for the prognostic classification of rectal cancer. Adapted from Jass, Love et al. (240)

Crohn's-like lymphoid reaction

It is recognised that the host anti-tumour immune response may manifest as nodular lymphoid aggregates deep to the invasive margin of the tumour in patients with colorectal cancer (Figure 1.9). Given its similarity to histopathological features identified in patients with Crohn's disease, Graham and Appelman termed this the Crohn's-like lymphoid reaction (CLR), and reported its semi-quantitative assessment as a potential good prognostic factor (245). Subsequent reports confirmed the presence of a conspicuous CLR as a prognostic factor, independent of both pathological and molecular characteristics of the tumour (201, 246). In addition, several groups have reported assessment of the CLR as a useful method for identifying MSI tumours, recommending further testing for those with a dense CLR (246, 247). However, similar to Jass criteria, difficulties with respect to its subjective nature and reproducibility have prevented mainstream acceptance.

Klintrup-Mäkinen grade

A more recently reported assessment of the peritumoural generalised inflammatory cell infiltrate is the Klintrup-Mäkinen (KM) grade (248). Based on semi-quantitative assessment, it describes not only the density of inflammatory cells at the invasive margin, but also the presence of active destruction of cancer cell islets by immune cells (Table 1.7, Figure 1.9). Detailed studies of the components of a weak and high KM grade have found an increase in the abundance of lymphocytes with increasing density (233), suggesting that it may be a useful surrogate for more detailed studies of the local inflammatory cell infiltrate and its nature.

The presence of a high KM grade has been validated as a predictor of improved survival in patients with node negative and node positive colorectal cancer (233, 243, 248, 249), and has been identified as a prognostic factor in a number of other solid organ cancers, including oesophageal and breast cancer (250, 251). In addition, assessment of KM grade

has been shown to have acceptable inter-operator variability (248). Given this, its reliance on routinely available tumour specimens, and its informative nature with respect to both the density and activity of the inflammatory cell infiltrate, it provides a useful tool which may be readily applied in routine clinical practice. Further prospective validation studies, particularly in the context of adjuvant therapy clinical trials are warranted.

Table 1.7 Klintrup-Mäkinen score and grade for the assessment of the generalised local inflammatory infiltrate at the invasive margin of colorectal cancer

Klintrup-Mäkinen score	Description	Grade
0	No increase in inflammatory cells at invasive margin	Low grade
1	Mild/patchy increase in inflammatory cells at invasive margin	
2	Prominent inflammatory reaction forming a band at the invasive margin	High grade
3	Florid cup-like infiltrate at the invasive edge with destruction of cancer cell islands evident	

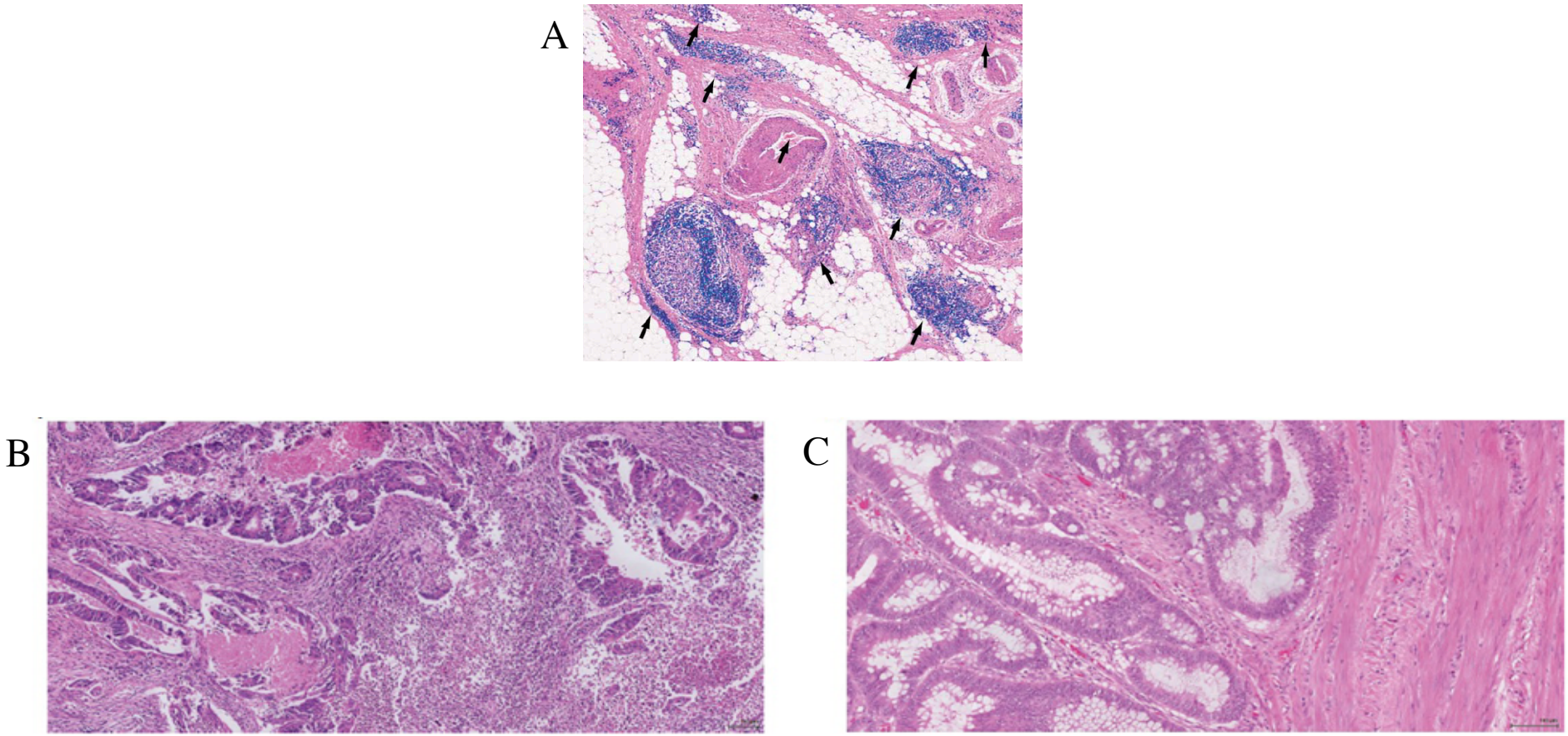


Figure 1.9 Examples of the generalised inflammatory cell infiltrate in colorectal cancer as examined using haematoxylin & eosin stained sections of the invasive margin. **(A)** Crohn's-like lymphoid reaction, **(B)** high Klintrup-Mäkinen grade displaying florid cup-like infiltrate at the invasive edge with destruction of cancer cell islands, and **(C)** low Klintrup-Mäkinen grade displaying no increase in inflammatory cells at the invasive margin. **(A)** adapted from Ueno, Hashiguchi et al. (252)

Assessment of the T-lymphocyte inflammatory infiltrate

Advances in immunohistochemistry and molecular techniques have led to increasing interest in the assessment of immune cell subsets within the local inflammatory cell infiltrate of patients with colorectal cancer (225). Of these, one of the most studied cell types has been tumour infiltrating T-lymphocytes. Composite scores, based on immunohistochemistry-based assessment of multiple T-lymphocyte subtypes, their density and their location within the tumour microenvironment have been proposed and have been reported as having superior prognostic value when compared to conventional TNM-based staging.

$CD3^+$ T-lymphocytes

Mature T-lymphocytes express CD3, and as such it has been one of the most widely reported markers of T-lymphocyte density in patients with colorectal cancer (225). Generally, $CD3^+$ T-cell density has been associated with increased survival and decreased disease recurrence. Several studies have identified associations between decreasing density of $CD3^+$ T-cells and the presence of adverse pathological characteristics such as increasing T stage, the presence of lymph node metastases, and venous, lymphatic and perineural invasion (227, 253). Furthermore, MSI status is strongly associated with $CD3^+$ density within the tumour microenvironment (254). Despite these associations, $CD3^+$ T-cell density has been shown to have prognostic value independent of such characteristics.

$CD8^+$ cytotoxic T-lymphocytes

Cytotoxic T-lymphocytes express CD8 and are an important mediator of the host cytotoxic response to pathogens and tumour cells. Multiple studies have identified the density of $CD8^+$ T-cells as an independent prognostic factor in patients with both localised and metastatic disease (225). In addition, several groups have reported the prognostic value of assessment of $CD8^+$ T-cell density in combination with other components of the tumour

microenvironment. For example, Lugli and colleagues reported that combined assessment of $CD8^+$ T-cells and tumour budding was an independent prognostic factor, and provided a more comprehensive representation of pro-tumour and anti-tumour characteristic than either measure alone (255).

$CD45RO^+$ memory T-lymphocytes

Once an immunological response to an antigen has occurred, immunological memory of the encounter is maintained by memory T-lymphocytes. These T-lymphocytes undergo a conformational change, expressing CD45RO and are able to mount a cytotoxic immune response much more rapidly on re-exposure. As such, their presence within the microenvironment is of considerable interest. Similar to other T-lymphocyte subsets, memory T-cell density is strongly associated with MSI status (256), likely due to the increased burden of tumour neo-antigens capable of eliciting immunological memory. Furthermore, an increased density of $CD45RO^+$ T-cells within the tumour microenvironment is associated with favourable pathological characteristics, such as less frequent lymphatic, perineural and venous invasion (227). Several studies have reported memory T-lymphocyte density as an independent prognostic factor in patients with colorectal cancer (225).

The Immunoscore

Although individual T-lymphocyte subsets have shown varying prognostic value in patients with colorectal cancer, it is increasingly apparent that assessment of a single immune cell type fails to encompass the complex immune and inflammatory milieu present within the tumour microenvironment. For this reason, several groups have proposed assessment of multiple immune cell types to provide more comprehensive characterisation. One such score is the Immunoscore proposed by Galon and Pagès (257), and based on the premise that it is not only immune cell type, but density and location within the tumour

microenvironment which are important determinants of the host anti-tumour immune response and therefore outcome.

Genomic studies of the tumour microenvironment by this group identified that the presence of a Th1-polarised adaptive immune response was associated with favourable outcome of patients with colorectal cancer (232). Furthermore, the presence of T-lymphocytes associated with a Th1-type response, namely those expressing $CD3^+$, $CD8^+$, $CD45RO^+$ and granzyme B^+, was associated with increased survival. Interestingly, combined assessment of multiple immune cell types and at different locations within the tumour microenvironment stratified survival greater than individual immune cell assessment within a single region, and had greater prognostic utility than conventional TNM-based staging. Indeed, the authors subsequently postulated that assessment of the 'immune contexture' as they termed it provides a comprehensive assessment of effective anti-tumour immunity, and therefore may more accurately predict patients at high risk of disease recurrence (258).

Further validation studies have confirmed the Immunoscore as a stage-independent prognostic marker in patients with colorectal cancer, including in the context of node negative disease (259, 260). Although originally proposed as an assessment of the density of cytotoxic and memory T-cells, difficulties with respect to staining and assessment of $CD45RO^+$ T-lymphocytes has led to subsequent revisions (257). The Immunoscore now reflects the density of $CD3^+$ and $CD8^+$ T-lymphocytes within the tumour core and invasive margin, providing five prognostic groups (Figure 1.10). A standardised protocol for the assessment of Immunoscore has been proposed and is now the subject of an ongoing, international validation study (257).

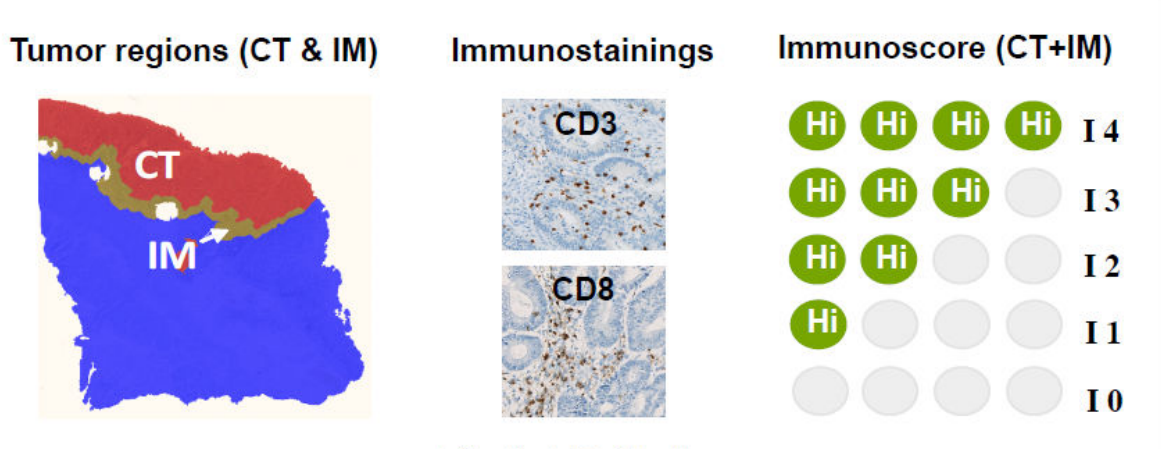


Figure 1.10 The Immunoscore – an assessment of $CD3^+$ and $CD8^+$ T-lymphocyte density within the tumour core (CT) and invasive margin (IM). Figure from Galon, Pagès, et al. (257)

The local inflammatory response and prognosis of patients with colorectal cancer – summary

Multiple studies have confirmed the local inflammatory cell infiltrate as a predictor of survival of patients with colorectal cancer. To this effect, numerous scoring systems, based on both assessment of the generalised inflammatory cell infiltrate as well specific immune cell subsets, have been proposed. However, the validity and reproducibility of these scores as well as their clinical utility in the routine staging of patients with colorectal cancer remain in question. The Immunoscore, an assessment of immune cell type, density and location, is currently the subject of an international validation study. However, whether such a score, reliant on immunohistochemistry with its inherent complications and added costs is superior to more comprehensive assessment of the generalised inflammatory cell infiltrate using routine pathological specimens, remains to be determined.

1.6.3 The systemic inflammatory response and prognosis of patients with colorectal cancer

The systemic inflammatory response is a normal physiological reaction, occurring as a rapid non-specific response to tissue injury. Complement and pentraxin activation, leukotriene and prostaglandin and pro-inflammatory cytokine release may all stimulate the

systemic inflammatory response as a result of tissue trauma or infection. What follows is a complex cascade of changes in circulating inflammatory cytokines and inflammatory and immune cell activity, with a number of local and systemic effects targeted at containing and eliminating the injuring stimuli and initiating tissue repair. In addition to initiating a stress response, with an increase in heart rate, blood pressure and catecholamine and steroid release, a number of other key physiological effects occur as part of the systemic inflammatory response (261) (Table 1.8). Although normally self-limiting due to endogenous anti-inflammatory cytokines, such as IL-10 (262), a chronic systemic inflammatory response may occur. In such circumstances, many of the physiological effects occurring are detrimental to host immune competence and may impact on recovery from both surgery and chemotherapy.

Table 1.8 Physiological effects of the systemic inflammatory response. Adapted from Gabay and Kushner (261)

System	Effects
Neuroendocrine	Fever, somnolescence, anorexia
	Increased secretion of endogenous steroids, vasopressin and catecholamines
	Decreased synthesis of insulin-like growth factor I
Haematopoietic	Anaemia
	Leukocytosis
	Thrombocytosis
Metabolic	Muscle loss
	Decrease gluconeogenesis
	Osteoporosis
	Cachexia
Hepatic	Changes in hepatic acute phase protein synthesis
	Changes in drug metabolism

The systemic inflammatory response in cancer

The systemic inflammatory response in malignancy is multifactorial in origin (263), involving tumour cell secretion of pro-inflammatory cytokines and activation of

inflammatory cells. Furthermore, tissue hypoxia is a common feature of many solid organ tumours and an important precipitant of the systemic inflammatory response in patients with cancer (264). In such circumstances, systemic inflammation favours tumour growth and dissemination by a number of mechanisms. As described above, these include promotion of tumour growth and angiogenesis through pro-inflammatory cytokine and growth factor secretion, subversion of host anti-tumour immunity, and facilitating tumour dissemination throughout the systemic circulation (263).

The presence of a measurable systemic inflammatory response is a marker of poor prognosis in patients with cancer and may drive many of the characteristics associated with advanced disease. Studies in patients with advanced malignancy identified the presence of an elevated systemic inflammatory response as being strongly associated with poor functional status, weight loss and reduced survival (265-267). Indeed, it is increasingly appreciated that chronic inflammation is a key component of the cancer cachexia syndrome (268), and may be a potential therapeutic target in its management (266).

Even in the context of non-metastatic disease, systemic inflammation is now recognised as an important determinant of survival. Independent of disease stage, the presence of a systemic inflammatory response has been associated with poorer survival (269). Furthermore, many of the physiological effects of inflammation may have detrimental effects following treatment, such as increased risk of complications following surgery, and impaired response to and tolerance of chemotherapy (270, 271).

Measuring the systemic inflammatory response in patients with colorectal cancer

Changes in circulating protein concentrations, known as the acute phase response, occur rapidly in the hours and days following initiation of the systemic inflammatory response (261) (Figure 1.11). The pro-inflammatory cytokine, IL-6, is the primary mediator of

many of these changes, influencing hepatic synthesis of acute phase reactants (272). In addition, changes in circulating immune cell numbers, particularly an increase in neutrophils and decrease in lymphocytes, are also evident. Such changes in inflammatory cell numbers and acute phase protein concentrations may be utilised to examine and monitor the systemic inflammatory response (271).

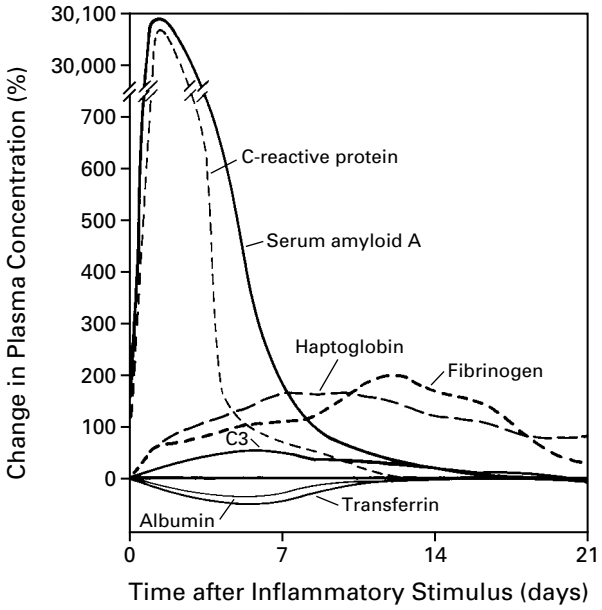


Figure 1.11 Changes in acute phase protein concentrations following initiation of a systemic inflammatory response. Figure from Gabay and Kushner (261)

Based on our knowledge of these biochemical and haematological parameters, a number of scores have been proposed to measure the cancer-associated systemic inflammatory response and predict survival (Table 1.9). One such score is the Glasgow Prognostic Score (GPS), a cumulative score derived from serum CRP and albumin concentrations (267). The prototypical acute phase protein, CRP, is a sensitive and routinely available marker of the systemic inflammatory response with demonstrable changes in serum concentrations (273). Similarly, serum albumin displays a clear change in circulating levels in the presence of a systemic inflammatory state. As such, the GPS offers a routinely available and simply calculated measure of the systemic inflammatory response with prognostic

value. This score was later revised to reflect the fact that hypoalbuminaemia in the absence of an elevated CRP did not reflect a chronic inflammatory state and was not associated with survival (269). Using the modified GPS (mGPS) it was possible to stratify cancer-specific survival of patients undergoing potentially curative resection of colorectal cancer from over 90% to approximately 50% at three years (269). Prognostic scores based on components of the differential white cell count have also been proposed. For instance, the neutrophil: lymphocyte ratio (NLR) (274), and the platelet: lymphocyte ratio (PLR) (275), have been proposed as inflammation-based prognostic scores in patients with cancer.

Table 1.9 Systemic inflammation-based prognostic scores in patients with cancer

Prognostic score	Components	Score
Modified Glasgow Prognostic Score	C-reactive protein ≤ 10mg/l and albumin ≥ 35g/l	0
	C-reactive protein > 10mg/l	1
	C-reactive protein > 10mg/l and albumin < 35g/l	2
Neutrophil: Lymphocyte Ratio	Neutrophil count : lymphocyte count < 5:1	0
	Neutrophil count : lymphocyte count ≥ 5:1	1
Platelet: Lymphocyte Ratio	Platelet count : lymphocyte count < 150:1	0
	Platelet count : lymphocyte count 150-300:1	1
	Platelet count : lymphocyte count > 300:1	2

The systemic inflammatory response and prognosis of patients with colorectal cancer – summary

The systemic inflammatory response represents one way by which the tumour may utilise normal, physiological host inflammatory responses to facilitate disease progression. In addition to underpinning many of the mechanisms responsible for tumour growth and dissemination, it is clear that a chronic systemic inflammatory response is also a key mediator of functional and nutritional decline in patients with advanced cancer. Furthermore, even in the context of potentially curative disease, assessment of systemic inflammatory profiles may aid in determining prognosis.

1.7 Local and systemic inflammatory responses as therapeutic targets in patients with colorectal cancer

Given the observed prognostic value of the host local and systemic inflammatory response in patients with colorectal cancer, therapeutic targeting presents an intriguing concept, particularly in patients with an "unfavourable" inflammatory profile. Immunotherapeutics, targeting host immunity to promote an effective anti-tumour immune response through several mechanisms, have been investigated in patients with colorectal cancer. Despite this, most studies to date have been in the context of metastatic, non-chemotherapy naïve patients, and have largely been limited to Phase II clinical trials with limited benefit.

However, it is increasingly apparent that other drugs, currently licensed for non-cancer indications, may have potentially favourable effects on cancer-associated inflammation, and thus may be repurposed for use in patients with cancer. Indeed, compared to novel agents, such drugs, including aspirin, NSAIDs, statins and histamine-2 receptor antagonists (H2RAs), provide an attractive option given their extensive safety profiles and relatively cheap cost. Furthermore, the wealth of data available from historical clinical trials as well as population-level studies has allowed post-hoc exploration of their potential chemotherapeutic role in patients with colorectal cancer.

Aspirin and NSAIDs, including COXIBs, have been identified as potential chemotherapeutic drugs which may favourably manipulate the inflammatory response in colorectal cancer. Despite convincing evidence from epidemiological studies and cardiovascular secondary prevention trials of a chemoprophylactic effect in reducing colorectal cancer incidence and mortality (276, 277), it is relatively recently that a potential benefit in patients with established disease has been realised, with NSAID users less likely to present with advanced or metastatic disease at diagnosis or follow-up (278, 279). Emerging evidence of as much as a 40% reduction in mortality in patients undergoing curative treatment makes the concept of the use of NSAIDs as adjuvant treatment in high

risk disease more compelling (70, 280-285). In such circumstances, the potential survival benefits may outweigh the risks which have so far precluded their use in CRC prevention (71).

Similarly, statins and H2RAs have also been identified as drugs with a potential benefit in improving survival and reducing risk of recurrence in patients with established colorectal cancer. A direct effect on tumour biology has been proposed through manipulation of several key signalling pathways, with a resultant effect on several of the key hallmarks of carcinogenesis, including proliferative and anti-apoptotic capacity as well tumour-mediated angiogenesis and invasiveness (210). Furthermore, these drugs have also been identified as potential agents capable of manipulating the host systemic and local inflammatory response to colorectal cancer (Table 1.10). Although the use of such drugs to manipulate the tumour and local and systemic environment in colorectal cancer presents an attractive concept, most evidence to date arises from *in vitro* and *in vivo* investigations, with little confirmation from clinical studies. In particular, there has been no attempt to stratify the use of anti-inflammatory agents and subsequent benefit in patients according to the presence of a systemic inflammatory response.

1.7.1 Immunotherapy

Efforts to employ immunotherapy in patients with colorectal cancer have concentrated on upregulating the host anti-tumour immune response. Over the past three decades, numerous treatment strategies have been employed; although cytokine therapy and toll-like receptor agonists have been investigated, most studies to date have examined cancer vaccines, adoptive cell transfer and immune checkpoint inhibition (286).

Vaccines

Cancer vaccines aim to upregulate anti-tumour immunity and cancer cell destruction by promoting the host's natural immune response to altered self-antigens, as loss of this normal physiological process is thought to contribute to immune evasion by cancer cells. Vaccines can be generated using several techniques which have shown varying success.

Autologous vaccines utilise tumour cells removed from the patient to ensure that all potential tumour-associated antigens are included (286). However, tumour cells also share numerous normal self-antigens which are present on non-malignant cells, thereby limiting efficacy. Other techniques to establish vaccines have been investigated, such as the use of specific short chain peptides, including CEA (287), to target tumour-specific antigens. Similarly, dendritic cell vaccines and viral or bacterial vectors have been utilised to increase immunogenicity (286). Most studies to date have been performed in patients with metastatic disease, with varying results. Of interest however, two phase III studies investigated the use of autologous cell vaccines in patients with stage II and stage III colon cancer. The results were conflicting; whereas Vermorken and colleagues found a reduced risk of recurrence in the study group, particularly in patients with stage II disease (288), the study by Harris and colleagues found no significant difference in survival (289). However, sub-group analysis of patients in the Harris study suggested a potential, albeit not statistically significant, improved survival in patients with a pronounced delayed cutaneous hypersensitivity reaction following repeated vaccination. It was suggested that this may reflect heterogeneity in vaccine quality within the study; however taking the presence of a cutaneous reaction as a surrogate for vaccine quality, the study did propose a benefit in patients who had been effectively immunised.

Adoptive cell transfer

Adoptive cell transfer utilises autologous T-lymphocytes which have been harvested from the tumour. These are then activated and the cell population expanded before transfusion. By activating the T-lymphocytes *ex* vivo, *in vivo* immune inhibition can be circumvented (290). Other techniques to enhance immunogenicity include genetically modifying cells to increase affinity for T-lymphocyte receptors, or depletion of regulatory T-lymphocytes with cyclophosphamide before cell transfusion to inhibit host immunosuppression. Although early phase I/II studies identified treatment toxicity as a significant concern (286), a more recent study of patients with metastatic colorectal cancer found good tolerability and a significant improvement in survival in treated patients (291).

Immune checkpoint inhibition

Immune checkpoint activation is one means by which the immune system confers tolerance against self-antigens (292). Recent studies of immunotherapy in patients with colorectal cancer have been founded on the premise that activation of these checkpoints by the tumour may circumvent host anti-tumour immunity.

Inhibition of two specific T-lymphocyte checkpoint receptors – CTLA-4, which prevents immune stimulation, and PD-1, which induces T-lymphocyte anergy, have been investigated in patients with colorectal cancer. Initial studies of a CTLA-4 inhibitor, tremelimumab, have shown little response (293). Conversely, a phase II study of pembrolizumab, a PD-1 inhibitor, observed improved progression-free survival in patients with MMR deficient metastatic colorectal cancer, but not those with MMR competent disease (294). This is consistent with studies which have identified immune checkpoint activation as a consistent feature of patients with MMR deficient tumours (295). Although suggesting a role for immune checkpoint inhibition in the treatment of patients with MMR

deficient colorectal, whether a subset of patients with MMR competent tumours will similarly benefit remains to be determined.

1.7.2 Aspirin, NSAIDs and COX-2 inhibitors

Recent evidence has suggested a potential beneficial effect of NSAIDs on colorectal cancer progression, with as much as a 40% reduction in cancer-specific mortality with regular aspirin and NSAID use (70, 282-285). Rothwell and co-workers suggested that the observed reduction in mortality apparent on secondary analysis of cardiovascular disease prevention trials was greater than what would be expected as a result of a NSAID-mediated decrease in cancer incidence alone (70). In addition, evidence that NSAID users are less likely to present with advanced or metastatic disease at diagnosis or follow-up further supports a direct effect on progression of established disease (278, 279).

Given such compelling evidence of an NSAID-mediated effect on established colorectal cancer, it is not surprising that their potential utility as adjuvant agents is currently being considered (281). Analysis of pre- and post-diagnosis NSAID usage further confirms a potential role for aspirin in addition to potentially curative surgery and adjuvant chemotherapy, with an almost 50% reduction in cancer mortality in patients who commence regular aspirin use following diagnosis (296). Interestingly, no significant survival benefit was seen in patients continuing pre-diagnosis aspirin use, suggesting that cancers arising in these circumstances may be aspirin-resistant (296, 297).

Surprisingly, there have been few trials of aspirin or NSAIDs as adjuvant agents in colorectal cancer. Sub-analysis of a randomised trial of 5-fluorouracil and leucovorin with or without irinotecan in patients with stage III colon cancer examined the effect of aspirin and COXIBs on recurrence and survival (298). Even after controlling for treatment arm, NSAID use was associated with a 50% reduction in disease recurrence or death. Two further clinical trials of adjuvant COXIB following curative resection in patients with stage

II/III disease ceased recruitment early following concerns regarding the cardiovascular safety profile of prolonged COXIB use (299, 300). The VICTOR trial, which randomised

Table 1.10 Direct tumour, local and systemic inflammatory effects of nonsteroidal anti-inflammatory drugs, statins and histamine-2 receptors antagonists in patients with colorectal cancer

Drug/Class	Direct tumour effects	Effects on local inflammatory response	Effects on systemic inflammatory response
NSAID	**Up-regulated:** • differentiation • apoptosis • cellular adhesion • radiosensitivity • susceptibility to oxidative stress	**Up-regulated:** • MHC class II expression • anti-tumour cytokines • inflammatory infiltrate • Th1/M1 response	**Up-regulated:** • lymphocyte and NK cell activity
	Down-regulated: • proliferation • angiogenesis • motility/migration	**Down-regulated:** • pro-tumour cytokines • COX-2 expression • T_{reg} activity	**Down-regulated:** • platelet activation • serum acute phase proteins
Statin	**Up-regulated:** • apoptosis • cell cycle arrest • susceptibility to oxidative stress • differentiation	Unknown effect on inflammatory infiltrate	
	Down-regulated: • proliferation • angiogenesis	**Down-regulated:** • NOS expression • COX-2 expression	**Down-regulated:** • circulating cytokines
H2 receptor antagonist	**Up-regulated:** • cellular adhesion • proliferation • angiogenesis	**Up-regulated:** • inflammatory infiltrate • anti-tumour cytokine • T_{reg} activity	**Up-regulated:** • lymphocyte and NK cell activity

patients who had undergone surgery and adjuvant treatment for stage II/III disease to daily rofecoxib or placebo, was terminated early with only 33% of patients receiving active treatment for at least one year (299). Interestingly however, despite no significant difference in cancer-specific mortality and recurrence-free survival, a statistically significant reduction in recurrence within the first year was found with regular COXIB use. Given that most adenoma prevention trials exposed patients to at least two years of regular COXIB use, the early termination of VICTOR likely precluded the investigators from finding any significant survival benefit.

Given the observed effects on tumour biology and microenvironment, the use of NSAIDs concomitant with neoadjuvant chemoradiotherapy has also been investigated. Indeed, decreased synthesis of protective prostaglandins via inhibition of COX-2 has been shown to increase tumour radiosensitivity (301). To date however, only phase II feasibility studies have shown a potential increase in tumour response and clinicopathological downstaging with the addition of COXIBs (302). Certainly such time-restricted use may be promising and favour the risk-benefit ratio of COXIB use. Regardless, although trials of adjuvant aspirin use are currently recruiting (303), it is clear that further, adequately powered trials are required to fully ascertain the benefit of aspirin, NSAIDs and COXIBS, both in the adjuvant and neoadjuvant setting.

Direct tumour effects

Pre-clinical investigations have found an increase in tumour cell apoptosis in association with a decrease in cell proliferation, angiogenesis and metastatic potential (304, 305). Although limited, mechanistic studies in patients with colorectal cancer have again suggested similar effects, with an NSAID-mediated decrease in primary and metastatic tumour blood flow and microvessel density even with short courses of NSAIDs (306, 307). Of further interest, NSAID administration has also been shown to facilitate tumour cell

differentiation, with a loss of cancer cell stemness and down-regulation of gene expression associated with increased metabolic turnover and resistance to oxidative stress (308, 309).

Cyclooxygenase-dependent effects

Several potential mechanistic pathways have been implicated in the anti-tumour effects of aspirin and other NSAIDs. The most studied mechanism is their inhibitory effect on COX-mediated synthesis of prostanoids, and in particular prostaglandin E_2 (PGE_2) (304, 305, 310, 311). Increased synthesis of PGE_2 by COX-2, the inducible form of the enzyme, has been shown to have several pro-tumour and immunosuppressant effects *in vitro* and *in vivo*, including an increase in tumour cell proliferation, decreased apoptosis, increased angiogenesis and increased chemo- and radio-resistance. Indeed, COX-2 is overexpressed in some but not all colorectal neoplasia, particularly those arising in the distal colon and rectum (312, 313), where its expression is associated with tumour invasiveness, metastatic potential and poorer survival (304, 310, 314). Furthermore, epidemiological evidence suggests a prominent role for COX-2 inhibition, with a reduced risk of COX-2 overexpressing tumours in long-term aspirin users and a modification of their anti-tumour effects observed in patients with common COX-2 gene polymorphisms (315, 316). Similarly, an increase in tumour cell apoptosis and decrease in tumour vascularity has also been confirmed in human subjects in response to NSAID administration, mediated by a reduction in COX-2 expression and tissue PGE_2 (306, 317).

Aspirin, particularly at low doses employed in cardiovascular disease, is a weak inhibitor of COX-2 whereas it remains a strong inhibitor of the constitutively expressed enzyme COX-1, particularly in anucleated cells such as platelets (318). As such, inhibition of COX-1 has also been suggested as another potential mechanism for the anti-tumour effects of NSAIDs by inhibiting platelet activation, facilitating immunosurveillance and preventing haematogenous spread. Indeed, aspirin can abrogate the increase in platelet

activation demonstrated in patients with colorectal cancer, even after only five days administration (319).

Cyclooxygenase-independent effects

Although many of the anti-proliferative effects of NSAIDs may be explained by their inhibitory effects on PGE_2 synthesis, several COX-independent actions have also been identified (320). Many of the effects of NSAIDs on proliferation and apoptosis have also been identified in cancer cell lines known not to express COX-2 (321). Several signal transduction pathways, including Wnt/β-catenin, NF-κB and the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway have been identified as potential targets for the non-COX mediated effects of NSAIDs, with limited clinical evidence suggesting an NSAID-mediated effect on associated signalling and transcription pathways (208, 321, 322). Epidemiological data again suggests these as valid targets of NSAID therapy in colorectal cancer, with increased survival associated with aspirin use in patients with *PIK3CA* mutated cancers (208), and a reduced risk of cancer with NSAIDs in patients with mutations within the NF-κB pathway (323).

Effects on cancer-related inflammation

The anti-inflammatory properties of aspirin and non-aspirin NSAIDs have identified them as likely candidates in the manipulation of cancer-associated inflammation; evidence of a NSAID-mediated attenuation of the acute phase response and weight loss in advanced cancer suggests a potential role in the management of the cancer cachexia syndrome. Furthermore, the chemoprophylactic effects of NSAIDs appear to be greater in patients with evidence of a systemic inflammatory response (64), although unfortunately, so do the cardiovascular risks of long-term COXIB use (324).

Local inflammation

The effects of aspirin, non-selective NSAIDs and COXIBs on the local inflammatory response have been investigated in a number of solid cancers, with significant anti-tumour responses identified in gastrointestinal, breast, bladder and head and neck cancers (325) (Table 1.11). A decrease in the levels of pro-tumour, immune-suppressing cytokines including PGE_2, has been identified in the colon and in colorectal hepatic metastases, likely mediated at a gene transcription level (306, 308, 317). Furthermore, NSAIDs have been shown to induce expression of MHC class II molecules on the surface of colorectal cancer cells (326). Such changes within the tumour microenvironment may in turn allow for the recruitment and propagation of a co-ordinated, effective anti-tumour lymphocytic response. Indeed, Lönnroth and colleagues have shown an increase in tumour infiltration of activated T-lymphocytes and a decrease in immunosuppressive regulatory T-lymphocytes following a short course of pre-operative indomethacin or celecoxib in patients with colorectal cancer (326). Similarly, indomethacin augmented the anti-CEA immune response *ex vivo* through inhibition of COX-2 and regulatory T-cell activity (327). The authors concluded that COX-2 inhibition could attenuate the inhibitory activity of regulatory T-cells identified in tumour tissue and regional lymph nodes, promoting an effective anti-tumour immune response. However, the longterm oncological benefits of NSAID-mediated manipulation of the local inflammatory response remain to be elucidated.

Systemic inflammation

The administration of NSAIDs has been shown to abrogate suppression of systemic lymphocyte and natural killer cell activity in patients undergoing major surgery (328, 329) and in patients with colorectal cancer (330, 331) (Table 1.12). NSAIDs attenuate the acute phase response in patients with advanced cancer, with a decrease in serum CRP identified in tandem with an improvement in weight and quality of life (266). Furthermore, the effect

of NSAIDs on reducing risk of colorectal cancer appears to be greatest in patients with evidence of systemic inflammation as measured by soluble tumour necrosis factor (TNF) receptor-2 (64). Interestingly however, in a polyp prevention study utilising low dose aspirin with or without folic acid, aspirin 325mg daily did not decrease CRP but did stabilise it over a three year period whereas patients receiving placebo experienced a significant increase (332). Regardless, CRP did not predict the chemoprophylactic effects of aspirin use. Despite this, the role of NSAIDs in patients with cancer-associated systemic inflammation undergoing potentially curative surgical resection remains largely unknown. In patients with rectal cancer, the use of celecoxib has been shown to decrease elevated circulating levels of TNFα and IL-8, potentially through a direct effect on tumour cells and NF-κB activity (333). Similarly, in patients with colorectal cancer and an elevated CRP, ibuprofen decreases circulating CRP, cortisol and IL-6 (334). Whether attenuation of the systemic inflammatory response by NSAIDs in patients with colorectal cancer undergoing curative surgery translates into a benefit in recurrence rates and survival however remains unknown, and must be addressed by future trials of neoadjuvant and adjuvant NSAID use.

1.7.3 Statins

Despite an unclear effect on the incidence of colorectal cancer, statins may influence the progression of established disease, with regular statin use being associated with earlier stage at diagnosis in three case-control studies (74, 335, 336). Siddiqui and co-workers, in a case-control study of 326 male users with colorectal cancer and regular statin use of at least three years, found a lower mean stage and lower frequency of metastases (28.4% vs. 38.8%, $P<0.01$) at presentation, with a higher prevalence of right-sided tumours in statin users (336). Furthermore, statin users had superior five-year survival (37% vs. 33%, $P=0.03$). Coogan and colleagues also found a significant reduction in the risk of stage IV disease (odds ratio (OR) 0.18, 95% confidence interval(CI) 0.05-0.62) with regular use of

statins for at least 3 months (335). Similarly, a modest reduction of stage III/IV disease was also observed by Poynter, however this failed to reach statistical significance (OR 0.90, 95% CI 0.54 to 1.50). In contrast however, despite finding a reduced risk of colorectal cancer with statin use, a recent case-control study from Scotland with prescription data linkage found no difference in stage at diagnosis or survival (76), although the study was underpowered to identify any significant survival benefit. Of more interest, a prospective observational study of statin use within a randomised trial of adjuvant chemotherapy in stage III colon cancer found no survival benefit with statin use, irrespective of duration of use or presence of *KRAS* mutations (337). These conflicting results may in part be explained by population-based genetic variation in HMG-CoA reductase, as the presence of single nucleotide polymorphisms have previously been hypothesised to modify the protective effect of statins on colorectal cancer risk (338).

It is clear that the benefit of statins in the treatment of colorectal cancer has not yet been defined and that further clinical trials are required. Recruitment for the National Surgical Adjuvant Breast and Bowel Project: Statin Polyp Prevention Trial is currently underway with the aim of investigating the effects of rosuvastatin on polyp/cancer recurrence and metachronous cancer development in patients who have undergone resection for stage I/II colon cancer (339)(335)(335)(325)(325)(313)(309)(307)(303)(298)(294)(294). This and further trials may in time define the role statins may play in treatment of colorectal cancer.

Direct tumour effects

Mevalonate, the end product of HMG-CoA reductase metabolism and its isoprenoid metabolites are required for the activation of the Ras superfamily of small GTPases by prenylation (340). In turn, these GTPases are crucial for downstream activity of several signal transduction pathways (341); inhibition of mevalonate synthesis by statins subsequently has indirect and direct effects on cell survival and growth. Such inhibition

has been shown to have a pleiotropy of effects, including a reduction in cell proliferation (78, 342), induction of apoptosis (78, 342), increased susceptibility to oxidative stress (343) and inhibition of metastatic transformation and angiogenesis (344). A role for non HMG-CoA reductase-mediated pathways has also been suggested, particularly in tumours exhibiting the CIMP phenotype. Hypermethylation of the bone morphogenic protein (BMP) pathway is common in CIMP tumours (345), and statin-mediated demethylation of the *BMP2* promoter region and subsequent BMP pathwa has previously been shown to increase apoptosis and promote cell differentiation in cell line studies (346). Indeed, such an effect may suggest a pertinent role for statins in patients with CIMP-associated tumours.

Of further interest, statin therapy has been shown to augment the activity of a number of chemotherapeutic agents, even in resistant cell lines (340, 347, 348). The activity of epidermal growth factor receptor inhibitors, including cetuximab, also appears to be potentiated *in vitro* and *in vivo*, even in cell lines with known *KRAS* mutations and resistance (349). Furthermore, statin therapy may also increase the likelihood of pathological complete response following neoadjuvant chemoradiotherapy(347, 350).

Effects on cancer-related inflammation

Cardiovascular disease prevention trials have identified a clear anti-inflammatory effect of statins, with down-regulation of pro-inflammatory cytokines and increased cardiovascular risk reduction in patients with elevated serum inflammatory markers (351). Furthermore, favourable effects on organ rejection following heart and renal transplant suggest a potent immunomodulatory effect, potentially through a direct effect on MHC class II expression and subsequent T-cell activation (352). Similar effects on the inflammatory response may also be expected in patients with colorectal cancer, and certainly evidence from clinical trials of a 90% reduction in risk of inflammatory bowel disease-related colorectal cancer is compelling (353).

Local inflammation

To date, no clinical evidence exists to support the role of statins in influencing the local inflammatory response in patients with colorectal cancer, although pre-clinical data suggests a direct inhibitory effect on NF-κB activation, with subsequent down-regulation of COX-2 and pro-inflammatory cytokine expression (354-356). A cohort study of patients undergoing radical prostatectomy found that statin use was associated with a reduced tumour inflammatory infiltrate (357); in contrast to colorectal cancer, however, a minimal local inflammatory response is associated with reduced recurrence and improved survival. Whether similar effects on the tumour inflammatory infiltrate in colorectal cancer can be expected remains to be seen.

Systemic inflammation

Despite a clear benefit on the systemic inflammatory response in cardiovascular disease and in patients following transplant, the clinical application of these effects in patients with colorectal cancer is less clear (Table 1.12). In an interventional study of patients undergoing curative resection, Malicki and co-workers found a significant reduction in pre-operative serum IL-6 in patients receiving statins (358). In contrast however, a recent study of the systemic inflammatory response to neoadjuvant chemoradiotherapy in patients with oesophageal and rectal cancer found that concomitant statin use did not attenuate the systemic inflammatory response or treatment-associated symptoms (359). Further clarification of the effects of statins on cancer-related systemic inflammation is required, and such measures should be incorporated in to future studies of the chemotherapeutic benefits of statins.

1.7.4 Histamine-2 receptor antagonists

Since early reports of a survival advantage in patients with gastric cancer (360), there has been interest in the potential use of H2RAs in the treatment of colorectal cancer. Aside

from potentially beneficial effects on the local and immune responses, pre-clinical data suggests direct anti-tumour effects, including inhibition of histamine as a growth factor and inhibition of tumour-endothelial cell adhesion and motility. Furthermore, prolonged H2RA use has been shown to increase the systemic bioavailability of 5-FU (361).

The first reports of a survival advantage for H2RAs in patients with colorectal were in the early 1990s, when Adams and co-workers reported a non-significant increase in three-year survival with peri-operative cimetidine in patients with Dukes' A to C disease (93% vs. 59%, *P*=0.17) (362). In 1995, Matsumoto and co-workers reported the survival analysis of a multicentre, randomised controlled trial of the effects of cimetidine on adjuvant 5-FUl-induced appetite loss and oesophagitis (363). Interestingly, they found a significant increase in survival for both colonic and rectal cancers at almost four years. A ten-year analysis from the same patient cohort further confirmed increased survival and reduced risk of recurrence with cimetidine, with greatest benefit seen in Dukes' C patients (364).

Further studies of differing doses and types of H2RAs given either prior to surgery or as adjuvant treatment have only shown a non-significant trend towards improved survival (365-368), particularly in patients with Dukes' C disease (365). Subgroup analyses have identified potential patient groups who may be more likely to benefit from H2RA treatment, such as those with MSS tumours or tumours with a low peritumoural lymphocytic infiltrate (366). As such patients with MSS tumours may represent a subgroup of patients likely to benefit from H2RA use, however no large scale studies have examined these relationships and therefore further investigation is necessary. In addition, patients who did not receive perioperative blood transfusion or develop post-operative infectious complications have similarly been identified as groups who may benefit oncologically (367). Differences in type and dose of drug used as well as inclusion of patients with metastatic disease at enrolment may have precluded finding significant results in these studies. The consistency of trend towards improved survival however does

suggest that further, standardised studies are required. A recent Cochrane Collaboration review of H2RAs as adjuvant treatment following colorectal cancer resection found overall a significant improvement in survival for cimetidine only (combined hazard ratio (HR) 0.53; 95% CI 0.32 to 0.87) (369). Given that most of the included trials were performed before the routine use of diagnostic cross-sectional imaging, total mesenteric excision surgery and contemporary chemoradiotherapy regimes, the authors advised caution regarding the applicability of these trials and advised the need for further studies incorporating current "best practice" treatment.

Direct tumour effects

Histamine acts as an autocrine tumour growth factor and has been shown to increase colorectal cancer cell proliferation and growth *in vitro* and *in vivo* (370). Indeed, expression of histamine and histidine decarboxylase, the enzyme responsible for histamine synthesis, is increased in cancer epithelium when compared to normal colorectal mucosa (371, 372); increasing expression has been associated with the presence of nodal and distant metastases as well as increased microvessel density, suggesting a potential role in the transformation to invasive and metastatic disease. Furthermore, histamine has also been shown to increase expression of COX-2 and PGE_2 as well as vascular endothelial growth factor in cell lines constitutively expressing COX-2 (371). Celecoxib has been shown to abrogate the histamine-induced increase in vascular endothelial growth factor expression, suggesting that at least some of the pro-tumour effects of histamine may be mediated by COX-2 and prostaglandin activity (371).

Although several histamine receptors have been identified with H2 and H4 receptor stimulation both being implicated in tumour growth (371), only H2 receptors appear to be preserved in colorectal cancer tissue with loss of H1 and H4 receptors when compared to normal mucosa (373). The use of H2RAs in both cell line and animal studies has been

associated with a decrease in histamine-induced tumour growth, proliferation and increase in apoptosis *in vitro* (370, 374). The use of H2RAs may also reduce the metastatic potential of colorectal tumour cells by inhibition of E-selectin expression, endothelial cell adhesion and a decrease in tumour microvessel density (364, 371).

Effects on cancer-related inflammation

Local inflammation

Activation of histamine receptor-2 on regulatory T-lymphocytes inhibits the cell-mediated immune response (375). Amelioration of this immunosuppressant effect by H2RA use has been shown to subsequently increase tumour infiltration of activated lymphocytes (Table 1.11). Adams and co-workers, using quantitative assessments of peri-tumoural lymphocytic infiltration such as the presence of a Crohn's-like reaction or Jass criteria, found an increased conspicuous lymphocytic infiltration with peri-operative cimetidine use (362, 376). Qualitative assessment of the lymphocytic infiltrate using immunohistochemistry have been equivocal, with one study suggesting that H2RA use increases tumour infiltration of $CD3^+$ T-lymphocytes, particularly in patients with late stage disease (377), whereas another study examining the dose-response of cimetidine suggested that H2RAs may exert their effects through other, non-$CD3^+$ cellular components (366). Interestingly, Kapoor et al. found that pre-operative use of the H2RA famotidine led to a significant increase in tumour lymphocyte infiltration in colon cancer rather than rectal cancer, with the largest effect seen in those patients with a normal pre-operative CEA (368).

Systemic inflammation

Histamine attenuates the systemic immune response in patients with colorectal cancer. Similarly, the exaggerated post-operative immune suppression experienced in patients with

colorectal cancer is in part mediated by histamine release (377). The use of H2RAs has been shown to abrogate tumour-associated systemic immune suppression, with restoration of circulating levels and activity of T-lymphocyte and natural killer cell subsets (378), potentially via augmentation of IL-2 and interferon activity (Table 1.13). Furthermore, peri-operative H2RA use restores normal cell-mediated immunity following surgery (377, 379). Although shown to decrease post-operative CRP in patients without cancer (380), the effects of H2RA use on systemic cytokine profiles and biomarkers of the systemic inflammatory response in patients with colorectal cancer remains unknown.

1.7.5 Local and systemic inflammatory responses as therapeutic targets – summary

Several strategies for targeting of the tumour-associated inflammatory response and host anti-tumour immunity have been investigated in patients with colorectal cancer. Early phase clinical trials have investigated the role of novel immunotherapy agents, including vaccines and monoclonal antibodies targeting immune checkpoints, with varying results. Such an approach is expensive, and significant drug toxicities have often hampered translation into phase III trials. Attempts to appropriately stratify patients, such as by MMR status, may aid in the identification of patients likely to benefit from novel therapies.

In spite of convincing epidemiological evidence, the role of statins, H2RAs and particularly NSAIDs in the management of patients with colorectal cancer has yet to be defined. Although shown to have a direct effect not only on tumour biology but also on the host systemic and local inflammatory response, most evidence has arisen from pre-clinical investigations *in vitro* and *in vivo*. The few clinical investigations described above have been limited in their clinical applicability, and the long-term oncological outcomes have not yet been fully explored.

The use of these agents is an attractive option not only because of their low cost, but also due to their relatively well-defined long-term safety profiles. Clinical trials of adjuvant aspirin and statins in patients with colorectal cancer are currently recruiting. Furthermore, their relatively common use in patients undergoing resection of colorectal cancer would allow for retrospective analysis to examine their effect on inflammatory profiles and outcome. It is clear however, that further studies are required to identify the role of anti-inflammatory agents in the management of patients with colorectal cancer, and particularly those patients identified at high risk due to the presence of an "unfavourable" inflammatory profile.

Table 1.11 The effects of nonsteroidal anti-inflammatory drugs and H2 receptor antagonists on the local inflammatory cell infiltrate of patients with colorectal cancer

Drug Class	Drug	Patient Group (*n*)	Study Type	Duration	Outcome Measure	Outcome	Comment
NSAID							
• **Lönnroth (2008)**	Indomethacin, Celecoxib	CRC (28) (1 Dukes D)	Randomised, controlled trial	3 days pre-op	TILs,	↑CD_4^+, CD_8^+ tumour infiltration ↓ T_{reg} tumour infiltration	
• **Yaqub (2008)**	Indomethacin	CRC (12) (5 Dukes D)	*Ex vivo* and histopathological study	N/A	Tumour and lymph node T_{reg} infiltration	↑tumour and lymph node infiltration by T_{reg}, COX-2 expression by lymph node T_{reg} ↓ T_{reg} activity *ex vivo*	NSAIDs may improve systemic and local immune responses by inhibiting circulating T_{reg} activity and COX-2 expressing T_{reg} cells identified in regional lymph nodes
H2 Receptor antagonist							
• **Adams (1994)**	Cimetidine	CRC (not given)	Randomised, controlled trial	7 days peri-op	TILs	↑tumour lymphocyte infiltration	Increased 3-year survival in cimetidine-treated patients (93% vs. 59%) associated with presence of lymphocytic infiltration
• **Adams (1997)**	Cimetidine	CRC (42) (8 Dukes D)	Randomised, controlled trial	5 days pre-op, 2 days post-op	Presence of "Crohn's-like reaction", Jass criteria, quantative assessment of "conspicuous lymphocyte response"	↑tumour lymphocyte infiltration using all measures	Presence of lymphocytic infiltrate associated with improved survival
• **Kelly (1999)**	Cimetidine (400mg and 800mg)	CRC (112) (22 Dukes D)	Randomised, controlled trial	5 days pre-op	Presence of "Crohn's-like reaction", Jass criteria, quantitatice assessment TILs, PTLs, CD_3^+, CD_8^+	↑ trend towards peritumoural lymphocytic infiltration in patients treated with cimetidine 800mg, no difference in CD_3^+, CD_8^+ between groups	Trend towards increased survival in group treated with cimetidine 800mg
• **Lin (2004)**	Cimetidine	Gastrointestinal cancers (38 CRC)	Randomised, controlled trial	7 days pre-op, 10 days post-op	TILs, PTL, CD_3^+, CD_{20}^+	↑tumour/peri-tumoural lymphocyte infiltration, predominantly CD_3^+ with few CD_{20}^+	Increase in TILs/PTLs even in patients with advanced stage disease (less likely to have pronounced inflammatory infiltrate)
• **Kapoor (2005)**	Famotidine	CRC (23) (2 Dukes D)	Randomised, controlled trial	7 days pre-op	TILs	↑in tumour lymphocyte infiltration	Greatest benefit seen in patients with colonic tumour and normal pre-operative CEA

↑ increased activity or expression in response to drug, ↓decreased activity or expression in response to drug, CRC – colorectal cancer, TIL – tumour infiltrating lymphocytes, PTL – peritumoural lymphocytes, CEA –carcinoembryonic antigen

Table 1.12 The effects of nonsteroidal anti-inflammatory drugs and statins on the systemic inflammatory response of patients with colorectal cancer

Drug Class	Drug	Patient Group (n)	Study Type	Duration	Outcome Measure	Outcome	Comment
NSAID							
• **Han (1983)**	Indomethacin	CRC (29) (11 patients Dukes C)	*Ex vivo*	N/A	MILPR	Increase in MILPR	MILPR impaired in up to 52% of CRC patients
• **Balch (1984)**	Indomethacin	CRC (57)	*Ex vivo*	N/A	MILPR	Increase in MILPR	MILPR impaired in CRC patients compared to controls;
• **McMillan (1995)**	Ibuprofen	CRC (9) (3 Dukes D)	Non-randomised, controlled study	8-11 days	Acute phase reactants	↓ CRP, IL-6, cortisol, platelet count No change in albumin, insulin, CEA, WCC	
• **Sciulli (2005)**	Aspirin	CRC (10)	Non-randomised, controlled study	5 days pre-op	Platelet activation	↓ COX-1 activity, platelet activity	Increase in platelet activation in CRC patients compared to controls
• **Konturek (2006)**	Celecoxib	Rectal (10)	Non-randomised, age- and sex-matched controls	14 days pre-op	Acute phase reactants, gastrin and progastrin	↓ TNFα, IL-8 serum and tumour gastrin, serum progastrin	Effects mediated by NFkB inhibition, increase in tumour COX-2 expression
• **Yaqub (2008)**	Indomethacin	CRC (12) (5 Dukes D)	*Ex vivo*	N/A	Anti-CEA immune response	↑anti-CEA immune response by inhibition of T_{reg} activity	
Statin							
• **Malicki (2009)**	Simvastatin	CRC (9)	Non-randomised, controlled study	14 days	Serum IL-6, IL-8	↓ IL-6, IL-8 (n.s.)	Elevated serum and tumour IL-6 and IL-8 in CRC patients compared to age-matched healthy controls.
• **Wang (2012)**	N/A	CRC undergoing CRT (50) (28% receiving statin)	Cohort study	N/A	Acute phase reactants, treatment-associated symptoms	Statin use did not influence acute phase reactants or symptom severity	

↑ increased activity or expression in response to drug, ↓decreased activity or expression in response to drug, CRC – colorectal cancer, MILPR – mitogen-induced lymphocyte proliferative response, CRT- chemoradiotherapy, CEA- carcinoembryonic antigen

Table 1.13 The effects of H2-receptor antagonists on the systemic inflammatory response of patients with colorectal cancer

Drug Class	Drug	Patient Group (n)	Study Type	Duration	Outcome Measure	Outcome	Comment
H2 Receptor antagonist							
• **Adams (1994)**	Cimetidine	CRC (50)	Randomised, controlled study	5 days pre-op, 2 days post-op	MILPR, CMI, lymphocyte subsets	No fall in MILPR or CMI compared to controls, No fall in B-cells in treatment group ↓ T-cells, NK cells in both groups.	
• **Nielsen (1995)**	Ranitidine	CRC (12 Dukes D)	*Ex vivo*	N/A	NK cell activity	Increased NK cell activity insignificantly, however augmented effect of IL-2 on NK cell activity	NK cell activity decreased in CRC compared to healthy volunteers (greater decrease in metastatic patients)
• **Lin (2004)**	Cimetidine	CRC (38)	Randomised, controlled study	7 days pre-op, 10 days post-op	Peripheral blood lymphocyte subsets (pre-op and post-op)	↑ CD_3^+, CD_4^+ and CD_4^+/ CD_8^+ ratio pre-operatively Improvement of post-op suppression of CD_3^+, CD_4^+ and CD_{57}^+	Peripheral blood CD_3^+, CD_4^+, CD_{57}^+ and CD_4^+/CD_8^+ ratio decreased in CRC patients compared to healthy controls and CD_8^+ increased

↑ increased activity or expression in response to drug, ↓decreased activity or expression in response to drug, CRC – colorectal cancer, MILPR – mitogen-induced lymphocyte proliferative response, CRT- chemoradiotherapy, CEA- carcinoembryonic antigen

1.8 Summary and Aims

1.8.1 Summary

Colorectal cancer is the second most common cause of cancer death in the United Kingdom and Western World. Although the landscape of diagnosis, treatment and ultimately prognosis has improved over the past few decades, it still remains the case that around half of patients undergoing potentially curative resection die within five years of diagnosis. Indeed, it is clear that further work is required to identify novel prognostic factors that may be utilised alongside current TNM-based staging. The local and systemic environment, encapsulating the host inflammatory response to cancer, represents two potential characteristics which may aid in the prognosis of patients with colorectal cancer.

It is increasingly appreciated that an elevated systemic inflammatory response is associated with poorer survival in a number of cancers, including colorectal cancer. Numerous inflammation-based prognostic scores, derived from acute phase reactants and components of the differential white cell count, have been proposed. The mGPS is one such measure and has been validated extensively in the literature as having stage-independent prognostic value in patients with colorectal cancer. However, it has not been established how this may be utilised alongside current TNM-based staging of patients undergoing potentially curative resection. Furthermore, although the mGPS has been validated internationally, it is not clear how the combination of TNM stage and a systemic inflammation-based score may stratify survival of patients from distinct geographical locations. Indeed, given that the systemic inflammatory response may influence response to chemotherapy, differences in systemic inflammatory profiles across distinct populations would be of considerable importance.

The local tumour environment is also of importance in determining both disease progression and outcome of patients with colorectal cancer. Numerous studies have

confirmed the value of measures of the local inflammatory cell infiltrate in determining prognosis, using both assessments of the generalised inflammatory cell infiltrate and the adaptive, T-lymphocytic response. It is now realised, however, that other components of the tumour microenvironment may similarly determine tumour biology and outcome. Despite this, it is not clear how these other components, such as the tumour-associated stroma, may relate to the local inflammatory cell infiltrate and other pathological features in patients undergoing resection of colorectal cancer. Furthermore, whether assessment of the tumour-associated stroma may determine survival independent of the local inflammatory response and other high-risk pathological characteristics remains to be determined.

Despite our understanding of the prognostic value of measures of the local and systemic environment, it is not fully understood what mechanisms underpin and potentially link them. Multiple tumour and host factors have previously been hypothesised, including age, comorbidity status, and the presence of tumour necrosis. Increasing tumour size and depth of invasion has previously been associated with the presence of a systemic inflammatory response and amelioration of the local inflammatory cell infiltrate. In keeping with this, it is not clear if such measures are simply reflective of increasing tumour invasiveness and its association with survival.

At the level of the cancer cell, it is known that molecular characteristics may determine host inflammatory responses and the local and systemic environment. With respect to the local inflammatory cell infiltrate, it is known that tumours arising via MMR deficiency/ MSI pathway elicit a pronounced local inflammatory cell infiltrate. However, the relationship between these tumour characteristics and the systemic inflammatory response has not yet been defined. In addition, whether the improved prognosis observed in patients with MSI colorectal cancers is truly independent of the local and systemic environment has not yet been established.

Activation of pro-inflammatory signal transduction pathways may also be responsible for determining the nature of the local and systemic environment. Indeed, numerous signalling pathways have been implicated not only in tumour-associated inflammation, but also in determining other tumour characteristics such as degree of invasiveness. One such pathway is the JAK/STAT3 pathway which is activated by the pro-inflammatory cytokine IL-6. However, although JAK/STAT3 activation has been associated with suppression of anti-tumour immune response in pre-clinical studies, it is not clear how cancer cell STAT3 expression may affect the local and systemic inflammatory response in patients with colorectal cancer.

Whereas the local inflammatory cell infiltrate and tumour-associated stroma have been shown to determine prognosis of patients with colorectal cancer when examined individually, it would be expected that a combined approach to assessment would be of greater value. Indeed, such an approach would provide a more holistic overview of the nature of the tumour microenvironment and would be expected to stratify survival greater than either measure alone. Given that both the generalised inflammatory cell infiltrate and tumour-associated stroma may be assessed using H&E-stained sections, this would be a compelling concept given its use of routine pathological specimens.

Although assessment of the generalised local inflammatory cell infiltrate using H&E-stained sections is attractive due to reliance on routine specimens and low associated costs, it is not yet clear how the prognostic value of more detailed measures of the inflammatory infiltrate compare. The Immunoscore, for instance, may provide more granularity with respect to survival, despite the inherent costs and complexities associated with immunohistochemistry. As such it remains imperative that these two differing techniques are compared, particularly in the context of assessment of other components of the local and systemic environment.

To date, most reported measures of the tumour microenvironment are performed using full sections following surgical resection of the tumour en bloc. Although this allows for comprehensive assessment, particularly at the invasive margin, it does preclude targeting of the tumour microenvironment in the neoadjuvant setting. Indeed, by identifying patients with an 'unfavourable' tumour microenvironment before surgery, it may be possible to target and 're-educate' both the local inflammatory cell infiltrate and the tumour-associated stroma in the neoadjuvant setting. Nearly all patients undergoing potentially curative resection of colorectal cancer will undergo colonoscopy and biopsy, thus providing an ideal opportunity to obtain pre-operative specimens for assessment of the tumour microenvironment. Whether biopsy-derived specimens are suitable, however, remains to be determined.

Finally, it is now accepted that anti-inflammatory drugs, such as aspirin, NSAIDs and statins, are associated with improved outcome of patients with colorectal cancer. Numerous mechanisms have been proposed, however given their intrinsic anti-inflammatory properties, it is likely that the favourable effect on tumour biology and outcome is in part due to mediation of the host inflammatory response. As such, it could be expected that patients receiving such drugs at the time of diagnosis would be less likely to exhibit evidence of a systemic inflammatory response. Indeed, it would be of interest to examine the potential role of the systemic inflammatory response as a potential predictive biomarker of response to anti-inflammatory drugs such as aspirin.

1.8.2 Hypothesis and aims

To address the above areas of uncertainty, two main hypotheses were proposed:

1. The local and systemic environment, as measured using components of the tumour microenvironment and systemic inflammatory responses, could provide additional prognostic value complimentary to present day, TNM-based staging of patients undergoing potentially curative resection of stage I-III colorectal cancer.

2. A number of tumour and host factors determine the local and systemic environment in patients undergoing potentially curative resection of stage I-III colorectal cancer, some of which may be potential therapeutic targets.

To examine these hypotheses, studies were performed in patients undergoing potentially curative resection of stage I-III colorectal cancer to achieve the following aims:

1. To examine the relationship between measures of the systemic inflammatory response, using the mGPS, TNM-based staging and survival.

2. To examine differences in the host and tumour characteristics associated with the mGPS in two geographically distinct populations.

3. To examine the relationship between the tumour-associated stroma, the local inflammatory response, host and tumour characteristics and survival.

4. To examine the relationship between MMR status, the local and systemic tumour environment and survival.

5. To examine the relationship between tumour cell STAT3 expression and the local and systemic tumour environment and survival.

6. To examine the clinical utility of a combined tumour microenvironment-based score, comprised of measures of the generalised inflammatory cell infiltrate and tumour-associated stroma.

7. To compared the prognostic value of measures of the generalised inflammatory cell infiltrate and T-lymphocytic infiltrate and tumour-associated stroma.

8. To examine the relationship between tumour invasiveness, the local and systemic environment and survival.

9. To examine the feasibility of pre-operative, colonoscopic biopsy-based assessment of the tumour microenvironment.

10. To examine the relationship between pre-operative aspirin and statin use, systemic inflammatory responses and host and tumour characteristics.

2 Colorectal cancer, systemic inflammation and outcome: staging the tumour and staging the host

2.1 Introduction

Colorectal cancer is the third most common cancer in the Western World and the second most common cause of cancer death (381). Prognosis and the need for adjuvant therapy is primarily based on pathological staging of the resected tumour using TNM criteria (382). However, such a scheme may fail to accurately distinguish patients at high risk of disease recurrence and death, particularly in the context of lymph node negative disease (383).

Characteristics pertaining to the host are also associated with outcome. For example, the presence of an elevated systemic inflammatory response, as evidenced by changes in circulating acute phase proteins or myeloid cells, is an important unifying host characteristic and has been consistently associated with reduced survival independent of stage across a number of cancers including colorectal cancer (384, 385). Systemic inflammation-based prognostic scores, such as the mGPS and the NLR have been validated to have prognostic value in a variety of operable cancers (384, 385). Of these, the mGPS, a cumulative score based on the presence of an elevated serum CRP and decreased serum albumin, has been reported to have superior prognostic value compared to the NLR in patients with operable colorectal cancer (386-389).

Although the prognostic value of the mGPS has been widely reported, how it might be incorporated into the existing TNM-based staging of colorectal cancer, and how it might be implemented into routine clinical practice is not clear. Therefore, the aim of the present study was to examine the clinical utility of the pre-operative mGPS in a large cohort of patients from a single institution undergoing potentially curative resection of colorectal cancer.

2.2 Patients and Methods

Patients from a single surgical unit at Glasgow Royal Infirmary (GRI) were identified from a prospectively collected and maintained database of elective and emergency colorectal cancer resections. Consecutive patients who had pre-operative measurement of serum CRP and serum albumin within 30 days prior to surgery and, who on the basis of preoperative abdominal computed tomography and laparotomy findings were considered to have undergone potentially curative resection of stage I-III colorectal adenocarcinoma between January 1997 and May 2013 were included. Patients with IBD-associated cancer, or cancer arising from the vermiform appendix were excluded. In addition, patients who underwent resection with palliative intent or local resection only were excluded.

Tumours were staged using the fifth edition of the TNM classification (382), with additional data taken from pathological reports issued following resection. Following surgery, all patients were discussed at a colorectal multidisciplinary meeting involving surgeons, oncologists, radiologists and pathologists with a colorectal cancer special interest; patients with stage III disease or high-risk stage II disease and no significant comorbidities precluding chemotherapy were offered primarily 5-FU-based adjuvant chemotherapy on the basis of current guidelines at the time.

Pre-operative serum CRP and albumin were recorded prospectively. Patients undergoing elective resection had CRP and albumin concentrations measured routinely within 30 days prior to elective surgery. In patients undergoing emergency resection, CRP and albumin measured on admission were recorded. The mGPS was calculated as follows: patients with a CRP ≤10mg/L were allocated a score of 0, a CRP >10mg/L a score of 1, and a CRP >10mg/L and albumin <35g/L a score of 2.

Patients were routinely followed up for five years following surgery with outpatient clinic review at three months, six months and then yearly until five years following resection.

Surveillance computed tomography was performed yearly during this period with regular colonoscopic surveillance. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that acted as the censor date. Cancer-specific survival was measured from date of surgery until date of death from recurrent or metastatic colorectal cancer. Overall survival was measured until the date of death from any cause.

Statistical Analysis

The relationship between mGPS and clinicopathological characteristics was examined using the χ^2 method for linear trend. The relationship between clinicopathological characteristics, pre-operative mGPS and survival was examined using Kaplan-Meier log-rank survival analysis and univariate Cox proportional hazards regression to calculate HRs and 95% CIs. Variables with a *P*-value ≤0.1 on univariate analysis were subsequently entered into a multivariate model using a backwards conditional method. Five and ten-year survival was presented as percentage of patients surviving (standard error (SE)). A *P*-value ≤0.05 was considered statistically significant for all analyses. All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). The West of Scotland Research Ethics Committee approved the study.

2.3 Results

One thousand patients who underwent potentially curative resection of colorectal cancer were studied. Clinicopathological characteristics are shown in Table 2.1. Data on neoadjuvant therapy, adjuvant therapy and tumour differentiation were missing in 19, two and 10 patients respectively. Two-thirds of patients were older than 65 at time of surgery, 55% were male and over 90% of patients underwent elective resection. Two thirds of patients underwent resection of colon cancer. Ninety-four patients with rectal cancer and five patients with colon cancer received neoadjuvant therapy prior to surgery; of these, thirteen patients with rectal cancer had complete pathological response (subsequently termed stage 0 disease). Overall, 15% of patients had stage I disease, 46% had stage II disease and 38% had stage III disease. A quarter of patients received adjuvant chemotherapy following surgery; 16% of patients with stage II disease and 45% of patients with stage III disease received adjuvant therapy.

Thirty-seven percent of patients had CRP >10mg/L and 26% had an albumin <35g/L prior to surgery. Almost two thirds of patients were mGPS=0, whereas 21% and 16% were mGPS=1 and mGPS=2 respectively. An elevated mGPS was associated with advancing age, emergency presentation (both $P \leq 0.001$), less frequent use of neoadjuvant therapy ($P<0.05$), colonic primary, advancing T stage, advancing TNM stage, poor tumour differentiation, surgical margin involvement, peritoneal involvement and tumour perforation (all $P \leq 0.001$) (Table 2.1).

The median follow-up of survivors was 56 months (range 10-206 months; interquartile range 28-107 months), with 242 colorectal cancer-related deaths and 193 non-cancer deaths. Cancer-specific survival at five and ten years was 75% and 67% respectively, and overall survival at five and ten years was 64% and 43%. The following clinicopathological characteristics were associated with reduced cancer-specific survival on univariate analysis

(Table 2.2): mGPS (P<0.001), advancing age (P<0.01), emergency presentation (P<0.01), T stage (P<0.001), N stage (P<0.001), poor differentiation (P<0.01), venous invasion (P<0.001), margin involvement (P<0.001) and peritoneal involvement (P<0.001). Tumour perforation showed a trend towards poorer cancer-specific survival (P<0.1). On multivariate survival analysis, mGPS was associated with reduced cancer-specific survival (HR 1.30, 95%CI 1.10-1.53, P=0.002), independent of age (P<0.01), T stage (P<0.001), N stage (P<0.001) and margin involvement (P<0.001). Poor differentiation and venous invasion showed a trend towards reduced survival on multivariate analysis (P=0.086 and P=0.084, respectively), whereas emergency presentation, peritoneal involvement and tumour perforation were not associated with survival.

The following clinicopathological characteristics were associated with reduced overall survival on univariate analysis (Table 2.2): mGPS (P<0.001), advancing age (P<0.001), emergency presentation (P<0.05), no adjuvant therapy (P<0.05), T stage (P<0.001), N stage (P<0.001), poor differentiation (P=0.001), venous invasion (P<0.01), margin involvement (P<0.001) and peritoneal involvement (P<0.001). On multivariate analysis mGPS was associated with reduced overall survival (HR 1.28, 95%CI 1.13-1.45, P<0.001), independent of age (P<0.001), adjuvant therapy use (P<0.05), T stage (P<0.05), N stage (P<0.001), differentiation (P<0.05) and margin involvement (P<0.001). Venous invasion showed a trend towards reduced overall survival (P=0.066), whereas emergency presentation, peritoneal involvement and tumour perforation were not associated with survival.

The relationship between pre-operative mGPS, TNM stage and cancer-specific survival is displayed in Table 2.3. Cancer-specific survival at five years varied from 100% in patients with stage 0 colorectal cancer to 61% in patients with stage III disease, and from 80% in patients with mGPS=0 to 61% in patients with mGPS=2 (Figure 2.1). When TNM stage and mGPS were combined, cancer-specific survival at five years varied from 100% in

patients with stage 0 disease and mGPS=0, to 32% in patients with stage III disease and mGPS=2 (P<0.001). A similar relationship between TNM stage, mGPS and ten-year cancer-specific survival was also observed; whereas survival ranged from 100% to 52% and from 70% to 52% with TNM stage or mGPS alone, the combination of TNM and mGPS stratified ten-year survival from 100% (TNM 0, mGPS=0) to 32% (TNM III, mGPS=2). The nature of the relationship between TNM stage and mGPS is shown for patients with TNM stage III disease in Figure 2.2 (P<0.001).

The relationship between pre-operative mGPS, TNM stage and overall survival is displayed in Table 2.4. TNM stage stratified survival at five years from 92% to 51%, and mGPS stratified survival from 70% to 46% (Figure 2.3). Ten year overall survival varied from 92% (stage 0) to 35% (stage III) and from 49% (mGPS=0) to 30% (mGPS=2). Combining TNM stage and mGPS, five-year overall survival ranged from 92% (TNM 0, mGPS=0) to 26% (stage III, mGPS=2) and ten-year overall survival ranged from 92% (TNM 0, mGPS=0) to 17% (TNM III, mGPS=2) (P<0.001). The effect of the combination of TNM stage and mGPS on overall survival is shown for patients with stage III disease in Figure 2.4 (P<0.001).

As mGPS was associated with emergency resection and a colonic primary, to control for any confounding of these variables the relationship between TNM stage, mGPS and survival was examined for 579 patients undergoing elective resection of colon cancer. In patients undergoing elective resection of colon cancer, an elevated mGPS was associated with advancing age, advancing T stage and TNM stage, poor differentiation, surgical margin and peritoneal involvement and tumour perforation (Table 2.5). The median follow-up of survivors was 58 months (range 10-206 months; interquartile range 28-107 months), with 122 cancer-related deaths and 124 non-cancer deaths. Cancer-specific and overall survival was 79% and 66% respectively at five years and 71% and 44% at ten years. On multivariate analysis, mGPS was associated with reduced cancer-specific

survival (HR 1.58, 95% CI 1.25-1.99, $P<0.001$), independent of age ($P<0.01$), T stage, N stage (both $P<0.001$) and margin involvement ($P<0.05$), and reduced overall survival (HR 1.53, 95% CI 1.30-1.81, $P<0.001$), independent of age ($P<0.001$), no adjuvant therapy ($P<0.05$), N stage ($P<0.001$) and margin involvement ($P<0.01$) (Table 2.6). Venous invasion, peritoneal involvement and tumour perforation were not associated with cancer-specific or overall survival on multivariate analysis.

In patients undergoing elective resection of colon cancer, cancer-specific survival at five years ranged from 96% in patients with stage I disease to 63% in patients with stage III disease and from 86% in patients with mGPS=0 to 65% in patients with mGPS=2 (Figure 2.5). Cancer-specific survival at ten years ranged from 96% (stage I) to 54% (stage III) and from 77% (mGPS=0) to 50% (mGPS=2). The combination of TNM stage and mGPS stratified both five and ten-year cancer-specific survival from 100% (stage I, mGPS=0) to 37% (stage III, mGPS=2) ($P<0.001$; Table 2.7).

The overall survival of patients undergoing elective resection of colon cancer was stratified by TNM stage from 79% to 53% at five years and from 46% to 38% at ten years, whereas mGPS stratified survival from 75% to 47% at five years, and from 54% to 24% at ten years (Figure 2.6). The combination of TNM stage and mGPS stratified overall survival at five years from 87% (stage I, mGPS=0) to 30% (stage III, mGPS=2) and at ten years from 55% (stage I, mGPS=0) to 17% (stage III, mGPS=2 ($P<0.001$; Table 2.8).

Subgroup analysis was subsequently performed to examine the relationship between mGPS, use of adjuvant chemotherapy and cancer-specific survival of 208 patients undergoing elective resection of stage III colon cancer. Use of adjuvant chemotherapy in patients with stage III colon cancer was associated with younger age ($P<0.001$), less advanced T stage and a lower mGPS (both $P<0.05$) but no other clinicopathological characteristics. The median follow-up of survivors was 62 months (range 11-205 months;

interquartile range 31-107 months), with 73 cancer-related deaths. Cancer-specific survival was 78% at five years and 63% at ten years for patients with stage III colon cancer who received adjuvant chemotherapy, compared to 51% and 47% respectively for patients who did not receive adjuvant therapy (*P*=0.003; Table 2.9). The mGPS stratified survival of patients with stage III colon cancer irrespective of adjuvant therapy status; for example, five-year survival varied from 91% (mGPS=0) to 57% (mGPS=1) for patients who received adjuvant therapy (*P*=0.002), and varied from 60% (mGPS=0) to 34% (mGPS=2) for patients who did not receive adjuvant therapy (*P*=0.117). Furthermore, whereas use of adjuvant therapy was associated with increased survival in patients with mGPS=0 (*P*=0.003), it was not associated with improved survival in patients with an elevated mGPS (*P*=0.431).

Finally, subgroup analysis was performed to examine the relationship between mGPS, ASCO high-risk pathological criteria (presence of a T4 tumour, lymph node yield <10 nodes, poor tumour differentiation, tumour perforation or venous invasion) and cancer-specific survival of 238 patients undergoing elective resection of stage II colon cancer without subsequent adjuvant therapy. The median follow-up of survivors was 64 months (range 10-205 months; interquartile range 28-111 months), with 39 cancer-related deaths. Five and ten-year survival of patients with no high-risk pathological characteristics was 91% and 85% respectively, compared to 85% and 72% for patients with one or more high-risk characteristic (*P*=0.212; Table 2.10). An elevated mGPS was associated with reduced survival of patients with both low and high-risk stage II colon cancer; ten-year survival of patients with low-risk disease was stratified from 88% (mGPS=0) to 68% (mGPS=2) (*P*=0.035), and ten-year survival of patients with high-risk disease varied from 72% to 53% (*P*=0.062).

2.4 Discussion

The results of the present study show how the combination of TNM and mGPS effectively stratifies outcome in patients undergoing potentially curative resection of colorectal cancer. These data support the routine staging of both the tumour and the host systemic inflammatory response in patients with colorectal cancer.

In the present study, an increasing mGPS was associated with the presence of high-risk clinicopathological characteristics pertaining to both the host and the tumour. Even so, the pre-operative mGPS was prognostic independent of TNM stage and routinely reported adverse tumour characteristics, such as peritoneal involvement and tumour perforation. Furthermore, although associated with emergency presentation and a colonic primary, which may potentially reflect site-specific tumour heterogeneity (390), it was of interest that the mGPS retained independent prognostic utility in the context of elective resection of colon cancer. The combination of TNM stage and mGPS increased the range of survival compared to either measure alone. For example, whereas five-year cancer-specific survival of all patients undergoing elective resection of stage III colon cancer was 63%, the addition of mGPS stratified survival from 75% to 37%. Furthermore, within stage II disease, it was possible to identify a fifth of patients undergoing resection at higher risk than that afforded by TNM criteria alone.

The present study was able to provide further insight regarding the relationship between systemic inflammatory responses and use of adjuvant chemotherapy for stage III colon cancer. Patients with an elevated mGPS prior to elective resection were less likely to receive adjuvant therapy. At the time of data collection, however, it was unlikely to have been a factor in the multidisciplinary team's decision to recommend chemotherapy. Furthermore, although an elevated mGPS was associated with advancing age, over 40% of patients who did not receive chemotherapy were younger than 75 at time of surgery. With

such observational studies, there is a concern that one might be examining a population with an associated but unrelated (to cancer) chronic inflammatory state, which may also be associated with a lower rate of adjuvant therapy. However, the common chronic inflammatory conditions, such as rheumatoid arthritis, do not normally preclude adjuvant chemotherapy. It is therefore of interest that an elevated mGPS has previously been associated with co-morbid status and the presence of post-operative infectious complications (391-393). However, although both may preclude use of adjuvant chemotherapy and explain the present inverse association between mGPS and use of adjuvant therapy (394), it is important to note that the relationship between mGPS and oncological outcome has previously been shown to be independent of underlying patient comorbidity (392, 395).

Of interest, the mGPS stratified the survival of patients who received adjuvant chemotherapy following resection of stage III colon cancer. Although the present analysis must be interpreted with caution, it is consistent with previous reports (384). Although patients with mGPS=0 had a 50% relative increase in survival at five years with adjuvant therapy, patients with mGPS≥1 appeared to derive no benefit. The underlying mechanism responsible for this lack of benefit is unclear, however may be orchestrated by inflammation-induced alterations in cytochrome-P450-mediated metabolism of chemotherapeutic drugs (396). Whereas it may be indicative of reduced tolerance to chemotherapy leading to subsequent dose reduction or cessation of treatment (397), it may also simply represent a lack of efficacy in the systemically inflamed patient. Certainly, although secondary analyses of reported trials of adjuvant chemotherapy may provide further insight, it is clear that future studies of adjuvant therapies should incorporate assessment of the pre-operative systemic inflammatory response.

Although there is clear rationale for the use of adjuvant chemotherapy in patients with stage III colon cancer, the post-operative management of lymph node negative disease is

problematic. Other high-risk pathological characteristics, such as the presence of venous invasion, have been shown to effectively stratify outcome within the confines of TNM staging, and may predict need for adjuvant therapy (179). However, the recent inclusion of venous invasion, alongside other high risk pathological characteristics as additional prognostic factors in tumour staging does not negate the utility of host characteristics, such as the mGPS, in the effective stratification of outcome. Indeed, in the present study, patients with mGPS=2 undergoing elective resection for otherwise low-risk stage II colon cancer had five and ten-year survival comparable to that of patients with stage III disease. Whereas assessment of pathological characteristics are often subjective and may be underreported (163), the components of the mGPS are objectively measured and routinely available. Although the small number of patients receiving adjuvant therapy for stage II colon cancer precluded meaningful analysis in the present study, whether the mGPS may aid in the selection of patients with stage II colon cancer likely to benefit from adjuvant therapy would be of considerable interest.

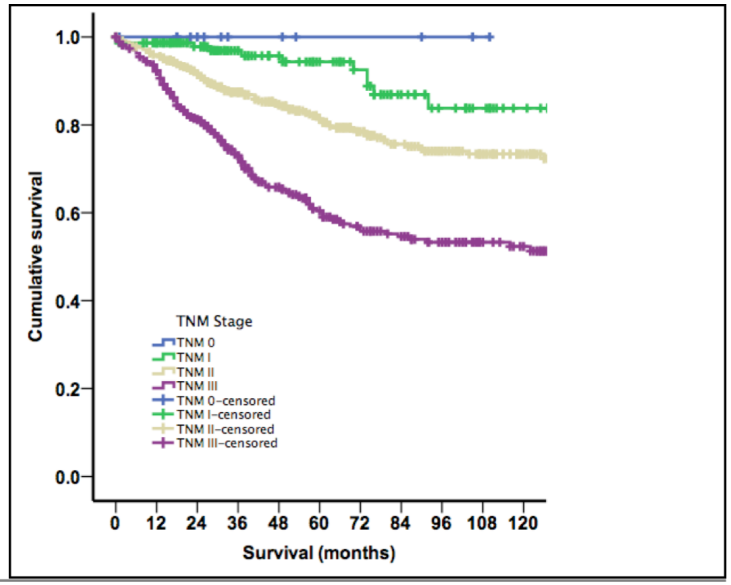
This study was limited by its single-centre nature; however, this was a large, prospectively collected cohort of patients. Although a population whose mGPS reverted to normal following surgery would be of interest, the majority of patients do not appear, in terms of their mGPS, to change their inflammatory state. Indeed of those patients with an elevated mGPS, up to 80% may remain systemically inflamed following potentially curative resection of colorectal cancer (388). As such, any changes to the operative and peri-operative management of patients over the time period studied, for example the introduction of enhanced recovery protocols to our centre in 2011, are unlikely to have had a significant effect on an elevated mGPS. Furthermore, the small number of patients undergoing resection for stage I colon cancer and patients with rectal cancer precluded meaningful analysis within these subsets. Finally, as mGPS was only recorded prior to

surgery, it was not possible to examine the impact of neoadjuvant chemoradiotherapy on the mGPS of patients with rectal cancer. This would also be of considerable interest.

Although representing only "the tip of a far larger iceberg" in inflammation-associated tumour progression and dissemination (398), the use of routinely available biomarkers, such as the mGPS, allows us to utilise our current understanding of the systemic inflammatory responses in patients with cancer. This has several far-reaching implications for clinical practice. As demonstrated, alongside guiding long-term prognosis, the incorporation of the mGPS into routine assessment may also identify patients less likely to tolerate, or benefit from, adjuvant systemic therapy. Furthermore, routine use of the mGPS may also direct future therapeutic strategies, targeted at the systemic inflammatory response itself. Indeed, it is now appreciated that systemic inflammation is complicit in cancer cachexia (399), and may be attenuated by the use of non-steroidal anti-inflammatory drugs (NSAIDs) (266). A similar scheme may also be applied to patients undergoing potentially curative surgery. For example, in patients with stage III disease, those with mGPS=0 may benefit from adjuvant chemotherapy alone, whereas those with an elevated mGPS may also benefit from the addition of an anti-inflammatory agent, such as aspirin or other NSAID (400). Certainly, it is clear that randomised controlled trials, incorporating both routine assessment of the systemic inflammatory response and use of anti-inflammatory agents, are required.

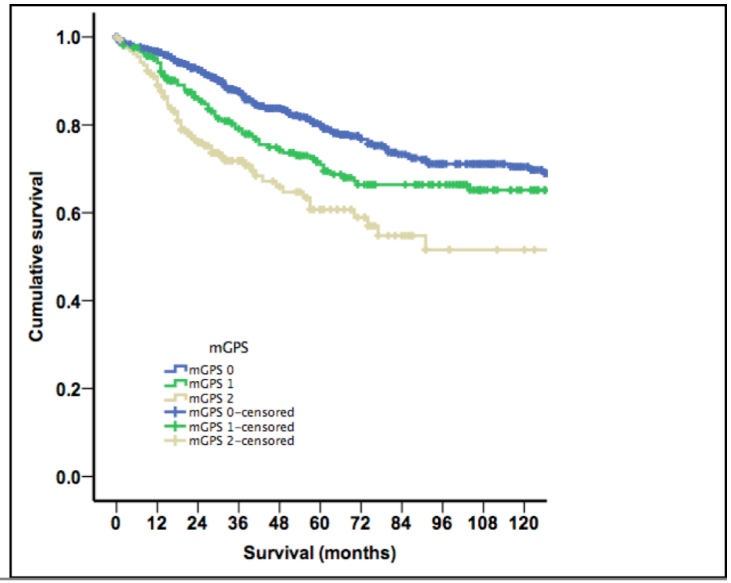
In conclusion, the mGPS provides complimentary prognostic information to current TNM-based staging and may also aid in directing future therapeutic strategies, targeting the systemic inflammatory response. Given that the combination of TNM stage and the mGPS are routinely available worldwide, this staging system for patients undergoing potentially curative resection of colorectal cancer has much to commend it.

A



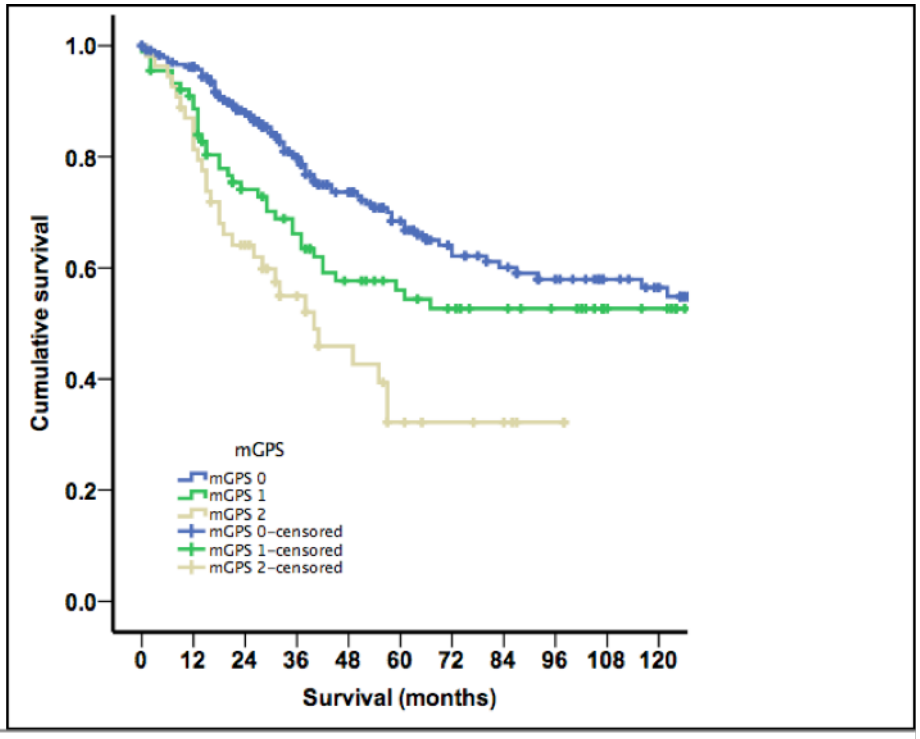
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
TNM 0	13	12	9	5	5	3	3	3	2	1	0
TNM I	148	139	115	85	71	59	50	35	25	19	12
TNM II	457	427	363	296	254	215	175	147	125	105	85
TNM III	382	346	268	208	163	126	102	90	76	58	51

B



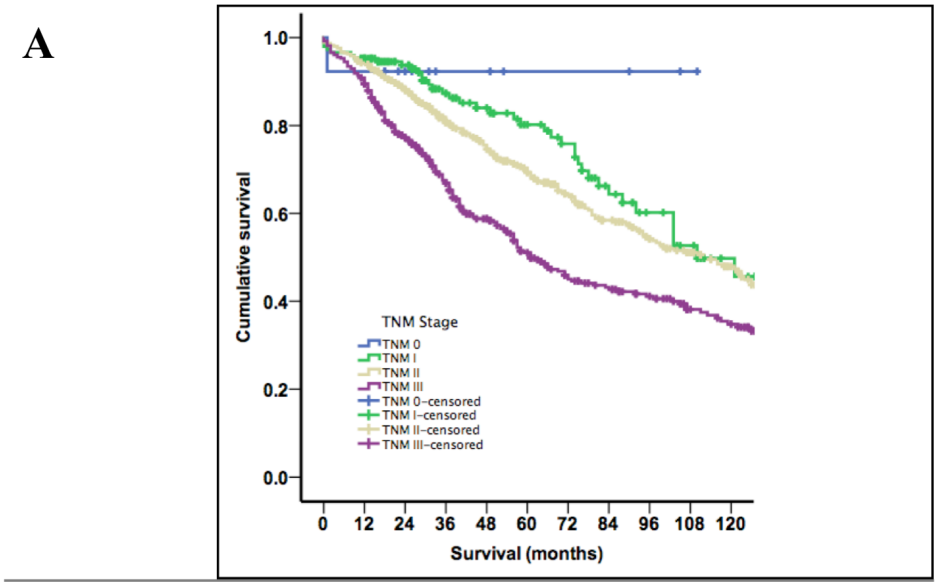
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	635	594	494	385	318	260	215	177	149	126	101
mGPS 1	207	191	158	136	121	100	83	76	65	45	36
mGPS 2	158	139	103	73	54	43	32	22	14	12	11

Figure 2.1 Ten-year cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer stratified by **(A)** TNM stage (log-rank $P<0.001$), and **(B)** modified Glasgow Prognostic Score (log-rank $P<0.001$)

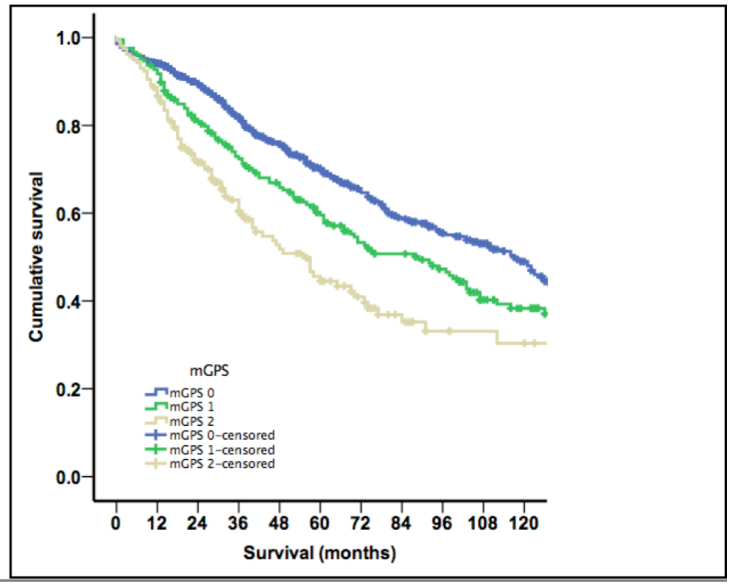


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	239	222	179	139	110	84	66	58	50	41	36
mGPS 1	89	78	57	49	39	34	30	27	24	17	15
mGPS 2	54	45	32	20	14	8	6	5	2	0	0

Figure 2.2 The relationship between modified Glasgow Prognostic Score and ten-year cancer-specific survival of patients undergoing potentially curative resection of stage III colorectal cancer (log-rank $P<0.001$)

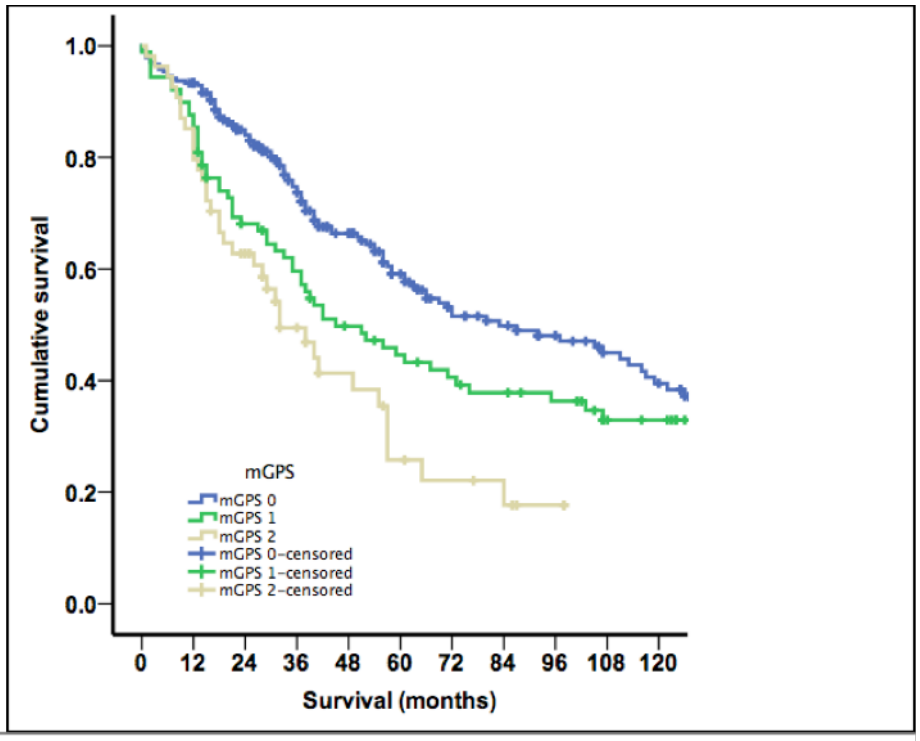


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
TNM 0	13	12	9	5	5	3	3	3	2	1	0
TNM I	148	139	115	85	71	59	50	35	25	19	12
TNM II	457	427	363	296	254	215	175	147	125	105	85
TNM III	382	346	268	208	163	126	102	90	76	58	51



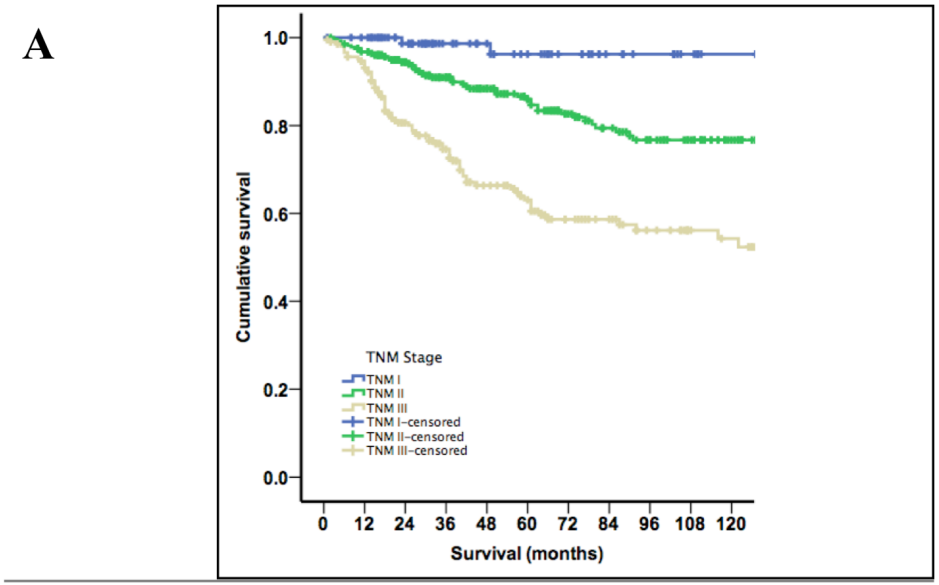
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	635	594	494	385	318	260	215	177	149	126	101
mGPS 1	207	191	158	136	121	100	83	76	65	45	36
mGPS 2	158	139	103	73	54	43	32	22	14	12	11

Figure 2.3 Ten-year overall survival of patients undergoing potentially curative resection of stage I-III colorectal cancer stratified by **(A)** TNM stage (log-rank $P<0.001$), and **(B)** modified Glasgow Prognostic Score (log-rank $P<0.001$)

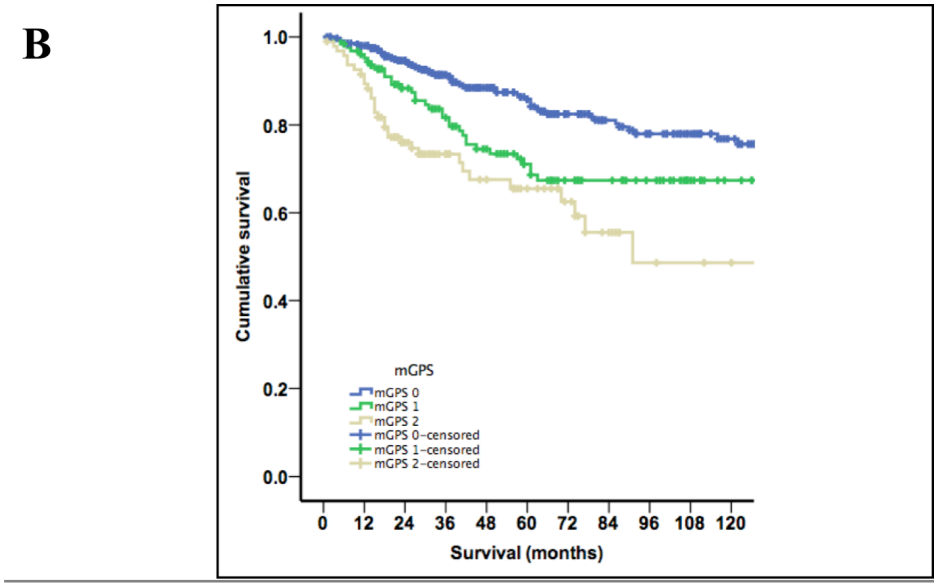


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	239	222	179	139	110	84	66	58	50	41	36
mGPS 1	89	78	57	49	39	34	30	27	24	17	15
mGPS 2	54	45	32	20	14	8	6	5	2	0	0

Figure 2.4 The relationship between modified Glasgow Prognostic Score and ten-year overall survival of patients undergoing potentially curative resection of stage III colorectal cancer (log-rank P<0.001)

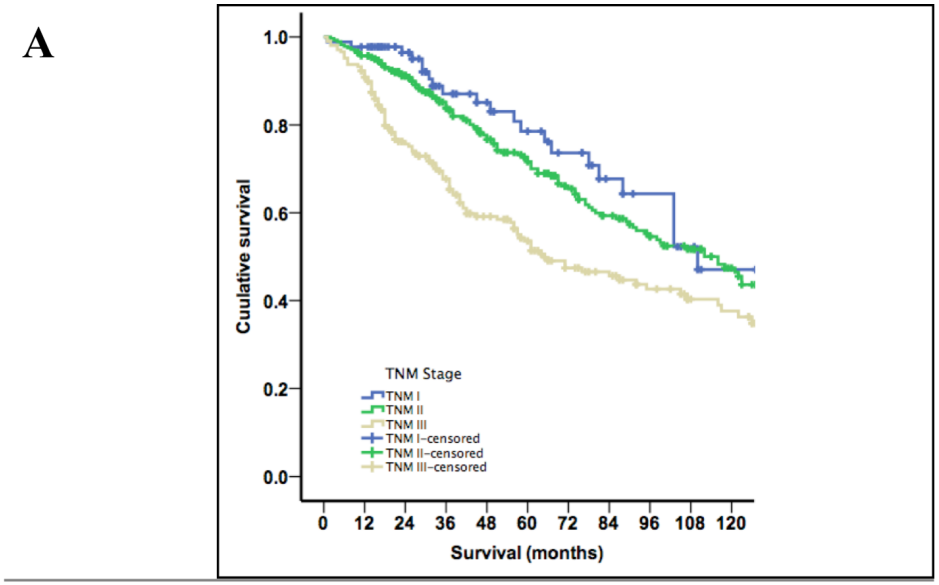


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
TNM I	89	86	72	49	42	35	28	19	16	11	7
TNM II	282	266	225	188	159	137	111	92	80	68	52
TNM III	208	191	140	115	90	74	57	51	40	31	28

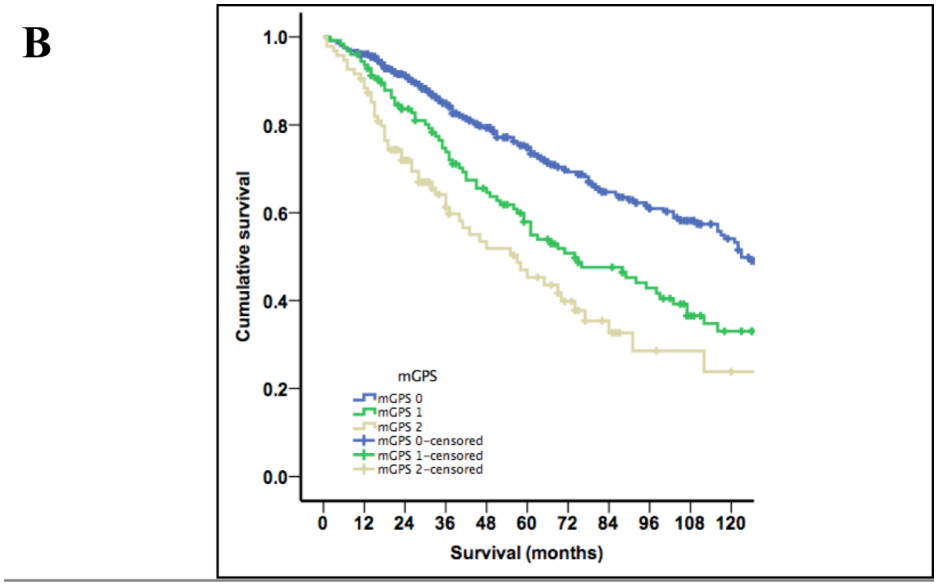


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	358	340	281	225	187	160	128	107	93	79	64
mGPS 1	126	118	96	83	70	58	48	43	36	25	18
mGPS 2	95	85	60	44	34	28	20	13	7	6	5

Figure 2.5 Ten-year cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colon cancer stratified by **(A)** TNM stage (log-rank $P<0.001$), and **(B)** modified Glasgow Prognostic Score (log-rank $P<0.001$)



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
TNM I	89	86	72	49	42	35	28	19	16	11	7
TNM II	282	266	225	188	159	137	111	92	80	68	52
TNM III	208	191	140	115	90	74	57	51	40	31	28



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
mGPS 0	358	340	281	225	187	160	128	107	93	79	64
mGPS 1	126	118	96	83	70	58	48	43	36	25	18
mGPS 2	95	85	60	44	34	28	20	13	7	6	5

Figure 2.6 Ten-year overall survival of patients undergoing elective, potentially curative resection of stage I-III colon cancer stratified by **(A)** TNM stage (log-rank $P<0.001$), and **(B)** modified Glasgow Prognostic Score (log-rank $P<0.001$)

Table 2.1 The relationship between modified Glasgow Prognostic Score and clinicopathological characteristics of patients undergoing potentially curative resection of stage 0-III colorectal cancer
Data analysed using χ^2 analysis for linear trend.

		All	mGPS=0	mGPS=1	mGPS=2	
Clinicopathological Characteristics (*n* when data missing)		***n*=1000 (%)**	***n*=635 (%)**	***n*=207 (%)**	***n*=158 (%)**	***P***
Age						0.001
	<65	330 (33)	218 (34)	66 (32)	46 (29)	
	65-74	347 (35)	238 (38)	73 (35)	36 (23)	
	>75	323 (32)	179 (28)	68 (33)	76 (48)	
Sex						0.137
	Female	452 (45)	274 (43)	102 (49)	76 (48)	
	Male	548 (55)	361 (57)	105 (51)	82 (52)	
Presentation						<0.001
	Elective	913 (91)	610 (96)	174 (84)	129 (82)	
	Emergency	87 (9)	25 (4)	33 (16)	29 (18)	
Neoadjuvant therapy						0.020
(981)	**No**	883 (88)	544 (88)	199 (97)	140 (91)	
	Yes	98 (10)	77 (12)	7 (3)	14 (9)	
Adjuvant therapy						0.805
(998)	**No**	750 (75)	483 (76)	145 (70)	122 (78)	
	Yes	248 (25)	151 (24)	62 (30)	35 (22)	
Tumour site						<0.001
	Colon	661 (66)	381 (60)	157 (76)	123 (78)	
	Rectum	339 (34)	254 (40)	50 (24)	35 (22)	
T stage						<0.001
	0	13 (1)	13 (2)	0 (0)	0 (0)	
	1	66 (7)	56 (9)	7 (3)	3 (2)	
	2	112 (11)	95 (15)	11 (5)	6 (4)	
	3	550 (55)	354 (56)	111 (54)	85 (54)	
	4	259 (26)	117 (18)	78 (38)	64 (41)	
N stage						0.470
	0	618 (62)	396 (62)	118 (57)	104 (66)	
	1	274 (27)	182 (29)	58 (28)	34 (22)	
	2	108 (11)	57 (9)	31 (15)	20 (13)	
TNM stage						0.001
	0	13 (1)	0 (0)	0 (0)	0 (0)	
	I	148 (15)	126 (20)	14 (7)	8 (5)	
	II	457 (46)	257 (41)	104 (50)	96 (61)	
	III	382 (38)	239 (38)	89 (43)	54 (34)	
Less than 10 lymph nodes retrieved						0.267
	No	824 (82)	518 (82)	171 (83)	135 (85)	
	Yes	176 (18)	117 (18)	36 (17)	23 (15)	
Tumour differentiation						<0.001
	Mod/well	894 (89)	584 (93)	181 (87)	129 (82)	
(990)	**Poor**	96 (10)	42 (7)	26 (13)	28 (18)	
Venous invasion						0.747
	No	493 (49)	312 (49)	108 (52)	73 (46)	
	Yes	507 (51)	323 (51)	99 (48)	85 (54)	
Margin involvement						0.001
	No	929 (93)	605 (95)	183 (88)	141 (89)	
	Yes	71 (7)	30 (5)	24 (12)	17 (11)	
Peritoneal involvement						<0.001
	No	773 (77)	531 (84)	138 (67)	104 (66)	
	Yes	227 (23)	104 (16)	69 (33)	54 (34)	
Tumour perforation						<0.001
	No	973 (97)	630 (99)	195 (94)	148 (94)	
	Yes	26 (3)	5 (1)	11 (6)	10 (6)	

Table 2.2 The relationship between clinicopathological characteristic and survival of patients undergoing potentially curative resection of stage 0-III colorectal cancer

	Cancer-specific survival				Overall survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.28 (1.09-1.50)	0.002	1.25 (1.07-1.47)	0.005	1.69 (1.50-1.91)	<0.001	1.57 (1.39-1.79)	<0.001
Sex (Female/ male)	1.08 (0.84-1.40)	0.534	-	-	1.14 (0.94-1.37)	0.189	-	-
Presentation (Elective/ emergency)	1.75 (1.20-2.55)	0.004	-	0.974	1.37 (1.01-1.88)	0.046	-	0.654
Neoadjuvant therapy (No/ yes)	1.16 (0.76-1.78)	0.485	-	-	0.84 (0.58-1.21)	0.349	-	-
Adjuvant therapy (No/ yes)	1.08 (0.81-1.44)	0.617	-	-	0.75 (0.59-0.96)	0.020	0.73 (0.56-0.95)	0.017
Tumour site (Colon/ rectum)	1.15 (0.89-1.95)	0.294	-	-	0.96 (0.82-1.22)	0.960	-	-
T stage (0/ 1/ 2/ 3/ 4)	1.98 (1.63-2.40)	<0.001	1.49 (1.21-1.83)	<0.001	1.37 (1.20-1.56)	<0.001	1.16 (1.01-1.33)	0.042
N stage (0/ 1/ 2)	1.88 (1.60-2.21)	<0.001	1.58 (1.33-1.88)	<0.001	1.42 (1.25-1.62)	<0.001	1.39 (1.21-1.60)	<0.001
Less than 10 lymph nodes retrieved (No/ yes)	1.28 (0.95-1.72)	0.110	-	-	1.15 (0.92-1.44)	0.227	-	-
Tumour differentiation (Mod-well/ poor)	1.81 (1.25-2.63)	0.002	-	0.086	1.63 (1.22-2.17)	0.001	1.36 (1.02-1.82)	0.038
Venous invasion (No/ yes)	1.69 (1.31-2.19)	<0.001	-	0.084	1.36 (1.12-1.65)	0.002	-	0.066
Margin involvement (No/ yes)	3.74 (2.67-5.23)	<0.001	2.61 (1.84-3.70)	<0.001	2.51 (1.87-3.36)	<0.001	2.06 (1.52-2.80)	<0.001
Peritoneal involvement (No/ yes)	2.12 (1.63-2.76)	<0.001	-	0.646	1.51 (1.22-1.86)	<0.001	-	0.733
Tumour perforation (No/ yes)	1.75 (0.93-3.29)	0.084	-	0.990	1.48 (0.88-2.47)	0.138	-	-
mGPS (0/ 1/ 2)	1.51 (1.29-1.76)	<0.001	1.30 (1.10-1.53)	0.002	1.43 (1.27-1.61)	<0.001	1.28 (1.13-1.45)	<0.001

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 2.3 The relationship between modified Glasgow Prognostic Score, TNM stage and five and ten-year cancer-specific survival of patients undergoing potentially curative resection of stage 0-III colorectal cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**
Stage 0	13	100 (0)	13	100 (0)	0	-	0	-
Stage I	148	94 (2)	126	97 (2)	14	72 (14) $^{0.008}$	8	-
Stage II	457	82 (2)	257	83 (3)	104	84 (4)	96	76 (5) $^{0.009}$
Stage III	382	61 (3) $^{<0.001}$	239	68 (4) $^{<0.001}$	89	56 (6) $^{0.001}$	54	32 (8) $^{<0.001/<0.001}$
Stage 0-III	1000	75 (2)	635	80 (2)	207	71 (3)	158	61 (5) $^{<0.001}$
	n	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**
Stage 0	13	100 (0)	13	100 (0)	0	-	0	-
Stage I	148	83 (5)	126	86 (5)	14	57 (17) $^{0.008}$	8	-
Stage II	457	73 (3)	257	75 (3)	104	76 (5)	96	61 (8) $^{0.0009}$
Stage III	382	52 (3) $^{<0.001}$	239	56 (4) $^{<0.001}$	89	53 (6) $^{0.001}$	54	32 (8) $^{<0.001/<0.001}$
Stage 0-III	1000	67 (2)	635	70 (2)	207	65 (4)	158	52 (6) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying cancer-specific survival within each TNM stage (rows) and prognostic value of TNM stage stratifying cancer-specific survival within each mGPS group (columns). CSS – cancer-specific survival, SE – standard error. Survival not calculated if $n<10$.

Table 2.3 displays the prognostic value of the combination of TNM stage and mGPS in determining five and ten-year cancer-specific survival relative to either measure alone in patients undergoing resection of stage 0-III colorectal cancer. For example, whereas five-year cancer-specific survival of patients with stage III disease was 61%, the addition of mGPS stratified survival from 68% to 32% ($P<0.001$). Similarly, whereas five-year cancer-specific survival of patients with mGPS=0 was 80%, the addition of stage stratified survival from 100% to 68% ($P<0.001$).

Table 2.4 The relationship between modified Glasgow Prognostic Score, TNM stage and five and ten-year overall survival in patients undergoing potentially curative resection of stage 0-III colorectal cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Stage 0	13	92 (7)	13	92 (7)	0	-	0	-
Stage I	148	80 (4)	126	87 (4)	14	59 (14) $^{0.006}$	8	-
Stage II	457	70 (2)	257	74 (3)	104	74 (5)	96	57 (6) $^{0.003}$
Stage III	382	51 (3) $^{<0.001}$	239	59 (4) $^{0.002}$	89	45 (5) $^{0.013}$	54	26 (7) $^{<0.001/\ 0.011}$
Stage 0-III	1000	64 (2)	635	70 (2)	207	60 (4)	158	46 (5) $^{<0.001}$
	n	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**
Stage 0	13	92 (7)	13	92 (7)	0	-	0	-
Stage I	148	49 (7)	126	56 (8)	14	16 (14) $^{0.006}$	8	-
Stage II	457	48 (3)	257	53 (4)	104	44 (6)	96	38 (7) $^{0.003}$
Stage III	382	35 (3) $^{<0.001}$	239	40 (4) $^{0.002}$	89	33 (5) $^{0.013}$	54	17 (7) $^{<0.001/\ 0.011}$
Stage 0-III	1000	43 (2)	635	49 (3)	207	38 (4)	158	30 (5) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying overall survival within each TNM stage (rows) and prognostic value of TNM stage stratifying overall survival within each mGPS group (columns). OS - overall survival, SE – standard error. Survival not calculated if $n<10$.

Table 2.4 displays the prognostic value of the combination of TNM stage and mGPS in determining five and ten-year overall survival relative to either measure alone in patients undergoing resection of stage 0-III colorectal cancer. For example, whereas five-year overall survival of patients with stage III disease was 51%, the addition of mGPS stratified survival from 59% to 26% ($P<0.001$). Similarly, whereas five-year overall survival of patients with mGPS=0 was 70%, the addition of stage stratified survival from 92% to 59% ($P=0.002$).

Table 2.5 The relationship between modified Glasgow Prognostic Score and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colon cancer
Data analysed using χ^2 analysis for linear trend.

		mGPS=0	mGPS=1	mGPS=2	
Clinicopathological Characteristic (*n* when data missing)		***n*=358 (%)**	***n*=126 (%)**	***n*=95 (%)**	***P***
Host characteristics					
Age					0.016
	<65	111 (31)	36 (29)	24 (25)	
	65-74	130 (36)	42 (33)	24 (25)	
	>75	117 (33)	48 (38)	47 (50)	
Sex					0.455
	Female	166 (46)	64 (51)	47 (50)	
	Male	192 (54)	62 (49)	48 (50)	
Neoadjuvant therapy					0.456
(569)	**No**	348 (99)	124 (98)	92 (99)	
	Yes	2 (1)	2 (2)	1 (1)	
Adjuvant therapy					0.663
	No	271 (76)	93 (74)	75 (79)	
	Yes	87 (24)	33 (26)	20 (21)	
Tumour characteristics					
T stage					<0.001
	1	33 (9)	2 (2)	2 (2)	
	2	53 (15)	6 (5)	3 (3)	
	3	195 (55)	70 (56)	53 (56)	
	4	77 (22)	48 (38)	37 (39)	
N stage					0.831
	0	231 (65)	75 (60)	65 (68)	
	1	99 (28)	35 (28)	21 (22)	
	2	28 (8)	16 (13)	9 (9)	
TNM stage					0.017
	I	77 (22)	8 (6)	4 (4)	
	II	154 (43)	67 (53)	61 (64)	
	III	127 (36)	51 (41)	30 (31)	
Less than 10 lymph nodes retrieved					0.374
	No	294 (82)	98 (78)	84 (88)	
	Yes	64 (18)	28 (22)	11 (12)	
Tumour differentiation					<0.001
(566)	**Mod/well**	332 (93)	109 (87)	71 (76)	
	Poor	24 (7)	17 (14)	23 (25)	
Venous invasion					0.765
	No	186 (52)	67 (53)	47 (50)	
	Yes	172 (48)	59 (47)	48 (51)	
Margin involvement					0.012
	No	349 (98)	117 (93)	88 (93)	
	Yes	9 (3)	9 (7)	7 (7)	
Peritoneal involvement					0.004
	No	287 (80)	83 (66)	66 (70)	
	Yes	71 (20)	43 (34)	29 (31)	
Tumour perforation					0.001
	No	356 (99)	120 (95)	90 (95)	
	Yes	2 (1)	6 (5)	5 (5)	

Table 2.6 The relationship between clinicopathological characteristics and survival of patients undergoing potentially curative, elective resection of stage I-III colon cancer

	Cancer-specific survival				Overall survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.41 (1.12-1.76)	0.003	1.39 (1.10-1.75)	0.005	1.87 (1.58-2.21)	<0.001	1.73 (1.46-2.06)	<0.001
Sex (Female/ male)	0.93 (0.65-1.33)	0.696	-	-	1.03 (0.80-1.32)	0.849	-	-
Neoadjuvant therapy (No/ yes)	0.87 (0.12-6.24)	0.891	-	-	0.41 (0.06-2.91)	0.371	-	-
Adjuvant therapy (No/ yes)	1.16 (0.75-1.67)	0.595	-	-	0.64 (0.46-0.89)	0.007	0.67 (0.46-0.97)	0.032
T stage (0/ 1/ 2/ 3/ 4)	2.48 (1.85-3.34)	<0.001	1.80 (1.30-2.49)	<0.001	1.38 (1.15-1.65)	0.001	-	0.339
N stage (0/ 1/ 2)	2.14 (1.70-2.69)	<0.001	1.88 (1.47-2.40)	<0.001	1.43 (1.20-1.72)	<0.001	1.63 (1.34-1.98)	<0.001
Less than 10 lymph nodes retrieved (No/ yes)	1.01 (0.65-1.57)	0.955	-	-	0.96 (0.71-1.31)	0.803	-	-
Differentiation (Mod-well/ poor)	1.59 (0.95-2.66)	0.075	-	0.668	1.59 (1.11-2.27)	0.012	-	0.226
Venous invasion (No/ yes)	1.83 (1.27-2.63)	0.001	-	0.256	1.29 (1.00-1.68)	0.050	-	0.335
Margin involvement (No/ yes)	4.18 (2.39-7.31)	<0.001	1.88 (1.05-3.48)	0.035	3.20 (1.99-4.99)	<0.001	2.16 (1.32-3.54)	0.002
Peritoneal involvement (No/ yes)	2.53 (1.77-3.62)	<0.001	-	0.988	1.45 (1.11-1.90)	0.007	-	0.275
Tumour perforation (No/ yes)	3.31 (1.46-7.54)	0.004	-	0.121	2.57 (1.32-5.02)	0.006	-	0.052
mGPS (0/ 1/ 2)	1.76 (1.42-2.18)	<0.001	1.58 (1.25-1.99)	<0.001	1.63 (1.40-1.91)	<0.001	1.53 (1.30-1.81)	<0.001

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 2.7 The relationship between modified Glasgow Prognostic Score, TNM stage and five and ten-year cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colon cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**
Stage I	89	96 (3)	77	100 (0)	8	-	4	-
Stage II	282	86 (2)	154	90 (3)	67	86 (5)	61	79 (6) $^{0.004}$
Stage III	208	63 (4) $^{<0.001}$	127	75 (4) $^{<0.001}$	51	52 (8) $^{0.001}$	30	37 (10) $^{<0.001/0.005}$
Stage I-III	579	79 (2)	358	86 (2)	126	71 (4)	95	65 (6) $^{<0.001}$
	n	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**
Stage I	89	96 (3)	77	100 (0)	8	-	4	-
Stage II	282	77 (3)	154	80 (4)	67	81 (5)	61	55 (11) $^{0.001}$
Stage III	208	54 (4) $^{<0.001}$	127	62 (6) $^{0.063}$	51	48 (8) $^{0.007}$	30	37 (10) $^{<0.001/0.041}$
Stage I-III	579	71 (3)	358	77 (3)	126	67 (5)	95	50 (8) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying cancer-specific survival within each TNM stage (rows) and prognostic value of TNM stage stratifying cancer-specific survival within each mGPS group (columns). CSS – cancer-specific survival, SE – standard error. Survival not calculated if $n<10$.

Table 2.8 The relationship between modified Glasgow Prognostic Score, TNM stage and five and ten-year overall survival of patients undergoing elective, potentially curative resection of stage I-III colon cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Stage I	89	79 (5)	77	87 (5)	8	-	4	-
Stage II	282	73 (3)	154	78 (4)	67	73 (6)	61	58 (7) $^{0.001}$
Stage III	208	53 (4) $^{0.001}$	127	66 (5) $^{0.063}$	51	39 (7) $^{0.007}$	30	30 (9) $^{<0.001/0.041}$
Stage I-III	579	66 (2)	358	75 (3)	126	58 (5)	95	47 (6) $^{<0.001}$
	n	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**	***n***	**10-yr OS % (SE)**
Stage I	89	46 (9)	77	55 (12)	8	-	4	-
Stage II	282	47 (4)	154	56 (5)	67	43 (7)	61	30 (9) $^{0.001}$
Stage III	208	38 (4) $^{0.001}$	127	50 (6) $^{0.063}$	51	25 (7) $^{0.007}$	30	17 (9) $^{<0.001/0.041}$
Stage I-III	579	44 (3)	358	54 (4)	126	33 (5)	95	24 (7) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying overall survival within each TNM stage (rows) and prognostic value of TNM stage stratifying overall survival within each mGPS group (columns). OS – overall survival, SE – standard error. Survival not calculated if $n<10$.

Table 2.9 The relationship between modified Glasgow Prognostic Score, adjuvant chemotherapy use and five and ten-year cancer-specific survival of patients undergoing elective, potentially curative resection of stage III colon cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**
Adjuvant therapy	96	78 (5)	64	91 (4)	24	57 (11) $^{0.002}$	8	-
No adjuvant therapy	112	51 (5) $^{0.003}$	63	60 (7) $^{0.003}$	27	47 (11) $^{0.798}$	22	34 (11) $^{0.117/0.591}$
All	208	63 (4)	127	75 (4)	51	52 (8)	30	37 (10) $^{<0.001}$
	n	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**
Adjuvant therapy	96	63 (7)	64	72 (9)	24	51 (12) $^{0.002}$	8	-
No adjuvant therapy	112	47 (6) $^{0.003}$	63	53 (8) $^{0.003}$	27	47 (11) $^{0.798}$	22	34 (11) $^{0.117/0.591}$
All	208	54 (4)	127	62 (6)	51	48 (8)	30	37 (10) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying cancer-specific survival within each adjuvant therapy group (rows) and prognostic value of adjuvant therapy use stratifying cancer-specific survival within each mGPS group (columns). CSS – cancer-specific survival, SE – standard error. Survival not calculated if $n<10$.

Table 2.10 The relationship between modified Glasgow Prognostic Score, American Society of Clinical Oncology high-risk pathological criteria and five and ten-year cancer specific survival of patients undergoing elective, potentially curative resection of stage II colon cancer

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS = 1		mGPS = 2	
	n	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**	***n***	**5-yr CSS % (SE)**
ASCO Criteria								
Low risk	91	91 (3)	48	93 (4)	30	100 (0)	13	68 (13) [0.035]
High risk[a]	147	85 (4) [0.212]	83	87 (5) [0.293]	28	84 (7) [0.647]	36	80 (8) [0.062/0.783]
All	238	87 (2)	131	89 (3)	58	92 (4)	49	77 (7) [0.002]
	n	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**	***n***	**10-yr CSS % (SE)**
Low risk	91	85 (5)	48	88 (6)	30	89 (7)	13	68 (13) [0.035]
High risk	147	72 (5) [0.212]	83	72 (7) [0.293]	28	84 (7) [0.647]	36	53 (14) [0.062/0.783]
All	238	77 (4)	131	79 (5)	58	87 (5)	49	56 (12) [0.002]

[a] High-risk stage II colon cancer denoted by presence of one or more of the following: T4 tumour, lymph node yield <10 nodes, poor tumour differentiation, tumour perforation or venous invasion. Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying cancer-specific survival within each ASCO risk group (rows) and prognostic value of ASCO risk group stratifying cancer-specific survival within each mGPS group (columns). CSS – cancer-specific survival, SE – standard error. Survival not calculated if $n<10$.

3 A comparison of the combination of TNM and the modified Glasgow Prognostic Score, and its association with survival of patients with colorectal cancer from United Kingdom and Japan

3.1 Introduction

In Chapter 2, it was observed that the mGPS held complimentary prognostic value to routine TNM-based staging of patients undergoing resection of TNM stage I-III colorectal cancer. Furthermore, the mGPS appeared to select for patients with stage III colon cancer less likely to derive benefit from adjuvant therapy. Therefore, it is of interest that the mGPS has been validated internationally in patients with colorectal cancer (384). Given its objectivity and potential role as a prognostic and predictive marker, the mGPS would be a useful adjunct to the routine staging of patients with colorectal cancer. Furthermore, similar to TNM-based staging, it offers the opportuinity to compare outcomes across the world by not only staging the tumour, but also the host.

However, systemic inflammation is determined by a number of host factors, including ethnicity; population studies have found those of Black and South Asian origin to have higher CRP concentrations than those of Caucasian descent (401-403), whereas individuals of East Asian heritage have consistently been reported as having significantly lower concentrations (404-406). Although studied in healthy subjects and cardiovascular disease screening programmes, it is not clear whether the presence of a cancer-associated systemic inflammatory response differs with ethnicity. Therefore, the aim of the present study was to examine the combination of TNM staging and the mGPS, and survival of patients from the United Kingdom (UK) and Japan undergoing potentially curative resection of TNM stage I-III colorectal cancer.

3.2 Patients and Methods

UK cohort

Patients from a single surgical unit at GRI, UK who underwent potentially curative resection of stage I-III colorectal adenocarcinoma from January 1997 to May 2013 without neoadjuvant chemoradiotherapy were identified from a prospectively collected database of elective and emergency colorectal cancer resections. Inclusion and exclusion criteria, pathological assessment of resected specimens, measurement of pre-operative CRP and albumin, and follow-up protocols are described in Chapter 2.

Patients were routinely followed up for five years following surgery, with surveillance computed tomography performed yearly with regular surveillance colonoscopy. Death records were complete until 30th May 2015 that acted as the censor date. Overall survival was measured from date of surgery until date of death from any cause. The West of Scotland Research Ethics Committee approved the study

Japanese cohort

Patients were identified retrospectively from a database of elective and emergency colorectal cancer resections performed by a single surgical team in the Department of Gastroenterological Surgery, Dokkyo Medical University, Japan (DMU). For the present study, patients who underwent potentially curative resection of TNM stage I-III colorectal cancer without neoadjuvant therapy between November 2005 to December 2015 were included. Exclusion criteria were identical to those applied to the GRI cohort, with pre-operative measurement of CRP and albumin performed on day of admission.

Patients were staged according to the seventh edition of the TNM classification (19). After discussion at colorectal multidisciplinary meetings, patients with stage III disease and

high-risk stage II disease were considered for 5-FU-based chemotherapy in accordance with current treatment guidelines.

Patients were routinely followed up for five years following surgery, with yearly surveillance computed tomography and regular surveillance colonoscopy (407). Deaths up until 30th April 2016 were included. Overall survival was measured from date of surgery until date of death from any cause, with survival censored at last clinic follow-up. The local institutional review board approved the study.

The mGPS for both cohorts was calculated as described in Chapter 2.

Statistical analysis

The relationship between study cohort, mGPS and clinicopathological characteristics was examined using the χ^2 method for linear trend. The relationship between mGPS and overall survival was examined using Kaplan-Meier log-rank analysis to calculate five-year survival percentage (SE). A *P*-value ≤ 0.05 was considered statistically significant. All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA).

3.3 Results

The study included 882 patients from GRI and 597 patients from DMU with stage I-III colorectal cancer. Data on American Society of Anesthesiologists (ASA) grade and adjuvant therapy use were missing for 298 and two patients respectively from GRI. Data on ASA grade, adjuvant therapy, lymph node yield, venous invasion, margin involvement and tumour perforation were missing for 38, 14, 6, 10 and 2 patients respectively from DMU.

A comparison of characteristics of the two cohorts is displayed in Table 3.1. Patients from GRI were more likely to be female, have a high ASA grade and present as an emergency, whereas patients from DMU were more likely to receive adjuvant chemotherapy and undergo resection for rectal cancer. Patients from GRI were likely to have more advanced disease than patients from DMU as measured by T stage, peritoneal and margin involvement, however patients from DMU were more likely to have evidence of venous invasion and tumour perforation. Lymph node yield and N stage were similar between groups. Patients from GRI were more likely to show evidence of an elevated mGPS (mGPS≥1: 39% vs. 16%, $P<0.001$). For subsequent analyses, only patients undergoing elective resection were included.

The relationship between mGPS and clinicopathological characteristics of patients undergoing elective resection of colorectal cancer is displayed in Table 3.2. In patients from GRI, mGPS was associated with advancing age and ASA grade, colonic primary, advancing T stage and TNM stage, peritoneal involvement and tumour perforation, and showed a trend towards an association with margin involvement. In patients from DMU, mGPS was associated with advancing age and ASA grade, colonic primary, advancing T stage and peritoneal involvement, and showed a trend towards an association with margin involvement. Although a similar trend between mGPS and TNM stage was observed in

patients from DMU, this did not reach statistical significance. The mGPS was not associated with use of adjuvant therapy, lymph node status, lymph node yield nor venous invasion in either cohort.

Comparison of the proportion of patients with an elevated mGPS in each cohort was subsequently performed after controlling for clinicopathological characteristics (Table 3.3). After controlling for age, sex, ASA grade and tumour location, patients from GRI were more likely to have an elevated mGPS compared to patients from DMU. Patients from GRI with T2-4 but not T1 disease were more likely to have an elevated mGPS than comparable patients from DMU. After controlling for N stage, TNM stage, venous invasion, lymph node yield and tumour perforation, patients from GRI were again more likely to have an elevated mGPS than patients from DMU.

The relationship between TNM stage, the mGPS and overall survival was examined (Table 3.4). The median follow-up of survivors from GRI was 76 months with 375 deaths and five-year overall survival of 65%. The median follow-up of survivors from DMU was 29 months with 53 deaths and five-year overall survival of 85%. TNM stage stratified five-year survival of patients from GRI from 80% to 53% (P<0.001) and patients from DMU from 94% to 84% (P=0.11); mGPS stratified survival of patients from GRI and DMU from 71% to 57% and 88% to 73% respectively (both P≤0.001). In the GRI cohort, the combination of TNM stage and mGPS stratified survival at five years from 85% (TNM stage I, mGPS=0) to 40% (TNM stage III, mGPS≥1) (P<0.001); in the DMU cohort, the combination of TNM stage and mGPS stratified survival at five years from 95% (TNM stage I, mGPS=0) to 68% (TNM stage III, mGPS≥1) (P<0.001).

As overall survival of patients from GRI was consistently lower than patients from DMU with comparable disease stage and systemic inflammatory profile, the relationship between TNM stage, mGPS and ASA grade was examined (Table 3.5). Even after controlling for

stage and mGPS, patients from GRI were likely to be ASA grade III/IV compared to patients from DMU.

To control for potential discrepancies in staging due to differences between the 5th and 7th edition of TNM staging, the relationship between T stage, the mGPS and survival was examined (Table 3.6). As there were few events in patients with T1 disease, patients with T1 and T2 disease were combined into one group. T stage stratified survival of patients from GRI from 79% to 52% (P<0.001) and patients from DMU from 93% to 71% (P=0.009). The combination of T stage and mGPS stratified survival of patients from GRI from 82% (T1-2, mGPS=0) to 46% (T4, mGPS≥1) (P<0.001), and patients from DMU from 95% (T1-2, mGPS=0) to 59% (T4, mGPS≥1) (P=0.002).

The relationship between mGPS and five-year overall survival of patients receiving adjuvant chemotherapy for TNM stage III colorectal cancer was examined (Table 3.7). An elevated mGPS was present in 37% of patients from GRI compared to only 11% of patients from DMU (P<0.001). The presence of an elevated mGPS stratified overall survival of patients from GRI from 75% to 51% (P=0.002), and patients from DMU from 85% to 68% (P=0.028).

3.4 Discussion

In the present study, systemic inflammatory profiles differed markedly in patients with colorectal cancer from distinct geographical locations, even after controlling for clinical and pathological characteristics. However, the combination of TNM stage and mGPS effectively stratified survival of patients with colorectal cancer in both cohorts. Within such TNM/mGPS-based stratification, there were significant differences in survival, with the Japanese cohort having superior overall survival. Therefore, the results of the present study not only show the clinical utility of TNM/mGPS-based staging, but also give an insight into the variation in outcomes for primary operable colorectal cancer worldwide.

What might explain the differences in survival between the UK and Japan? The basis of the differences in survival even after stratifying by TNM/mGPS may in part reflect differences in staging, with patients from the UK being effectively "understaged" compared to those from Japan. However, migration from the 5th to 7th edition would account for an upstaging from node negative to node positive disease in less than 3% of cases, with little prognostic implication (408, 409). Furthermore, differences in survival were still evident when patients were stratified by T stage, the definition of which has remained relatively stable through updates of TNM staging (410).

Another explanation is that patients from GRI were more likely to exhibit adverse host characteristics, including a higher burden of comorbidity as evidenced by ASA grade. Comorbidity and physiological status are independent determinants of survival, and may explain the differences observed in a cohort of patients otherwise comparable by TNM stage and systemic inflammatory profiles (391). Although it would be of interest to examine more objective measures of comorbidity (392), the present results highlight the importance of assessment of not only tumour, but also host characteristics when comparing outcomes.

Even after controlling for clinicopathological characteristics, patients from the UK were more likely to exhibit an elevated mGPS. This is consistent with previous studies which have identified lower circulating CRP concentrations in healthy individuals of East Asian origin compared to those of European descent (404-406, 411). Although the increased prevalence of an elevated systemic inflammatory response may be explained by differences in socioeconomic and lifestyle characteristics, markers of inflammation differ widely in individuals of different ethnicity resident in the same geographical location, thereby limiting the role of environmental factors and implicating other, intrinsic, factors (403, 411).

Circulating CRP levels are partly determined by genetic polymorphisms (412). Several associated single nucleotide polymorphisms (SNPs) have been identified (413-415), with a difference in not only their prevalence, but also their subsequent effect on CRP concentrations across different ethnic populations (415). A number of these SNPs have been confirmed as potential determinants of CRP concentrations in individuals of Asian descent (414), and the differences observed presently may reflect such underlying genetic polymorphisms. However, previous studies have generally considered mean population CRP concentrations in the region of 1-5mg/L, rather than >10mg/L as in the present study. Furthermore, in a prior study of patients with advanced cancer, no relationship between a number of candidate SNPs associated with inflammation and elevated CRP concentrations in the context of the cancer cachexia syndrome were identified (416).

Other factors therefore may be responsible for the differences observed. Comorbidity burden is associated with the presence of a systemic inflammatory response in patients with cancer (391, 392), however differences persisted even after controlling for ASA grade. Other factors, unmeasured in the present study, are as such likely to be implicated. One such factor is obesity, which is recognised as an important contributor to the systemic inflammatory response (417). Indeed, it is estimated that almost one quarter of males aged

20 or older are obese in the UK, compared to less than 5% in Japan (418). Therefore, it is likely that obesity and other uncontrolled factors, such as smoking, have contributed to the differing prevalence of elevated mGPS observed.

Despite differences in prevalence, the mGPS was associated with overall survival of patients from both the UK and Japan. The mGPS provided complimentary prognostic value to standard, TNM-based staging; it was possible to stratify survival of patients from both centres with node negative and node positive disease into low- and high-risk groups. Similarly, when utilising T stage alone, the mGPS again provided additional prognostic utility. Given its objectivity and reliance on routinely available serum markers, the results of the present study would further support the routine reporting of the mGPS as a prognostic marker in patients with primary operable colorectal cancer.

Consistent with the results of Chapter 2, an elevated mGPS was associated with reduced survival of patients receiving adjuvant chemotherapy for TNM stage III disease, however it was not possible to gain further insight into the mechanisms underlying this relationship. The present study only identified whether patients were commenced on adjuvant therapy, and did not consider either treatment duration or subsequent dose reductions. Such factors, and their relationship to systemic inflammation and outcome, are of considerable interest, and worthy of further study.

The present study has a number of limitations. Due to differences in cancer follow-up and attainment of mortality data, it was only possible to robustly report overall survival rather than cancer-specific or disease-free survival. However, reporting of overall survival provides a pragmatic measure of relevance to patients, and is increasingly recognised as a valuable metric for measuring outcome (419). In addition, the present study did not consider tumour molecular characteristics, such as mismatch repair deficiency (dMMR). However, few molecular characteristics, except for dMMR, *KRAS/BRAF* status have

translated into routine practice. Furthermore, in patients with non-metastatic disease, such measures are not used in the determination of prognosis or treatment in either institution. Finally, although the present study has examined two patient cohorts from geographically distinct locations, the ethnicity of individual patients was not considered. However, the population covered by GRI is predominantly European, with a small non-White population (420). Similarly, Japan has a predominantly East Asian population (421). Therefore, this is unlikely to have confounded results. Further confirmation of the results of the present study, particularly with respect to the differing prevalence of elevated systemic inflammatory responses in distinct geographical populations is required.

The results of the present study identify two intriguing points for further consideration. Firstly, the difference in systemic inflammatory profiles between geographically distinct populations raises issue with respect to the reporting of colorectal cancer outcomes. Whereas TNM staging has been standardised internationally to aid in the recruitment to and reporting of clinical trials, it is clear that similar must now occur with respect to the systemic inflammatory response. Second, given the perceived lack of efficacy of adjuvant chemotherapy in the systemically inflamed patient, such measures should be routinely reported to allow appropriate interpretation of clinical trial data.

In conclusion, using two geographically distinct populations, the results of the present study confirm the validity of the mGPS as a prognostic and potential predictive marker. Alongside tumour characteristics, such measures should be considered in future studies reporting outcome of patients undergoing resection of primary operable colorectal cancer.

Table 3.1 Comparison of clinicopathological characteristics of patients from Glasgow Royal Infirmary and Dokkyo Medical University undergoing potentially curative resection of stage I-III colorectal cancer

		GRI cohort	DMU cohort	
Clinicopathological Characteristics (*n* when data missing)		**(*n*=882) (%)**	**(*n*=597) (%)**	***P***
Host characteristics				
Age				0.146
	<65	279 (32)	219 (36)	
	65-74	293 (33)	176 (30)	
	>75	310 (35)	202 (34)	
Sex				0.003
	Female	402 (46)	226 (38)	
	Male	480 (54)	371 (62)	
ASA grade				<0.001
(1143)	**I/II**	315 (54)	490 (88)	
	III/IV	269 (46)	69 (12)	
Presentation				<0.001
	Elective	796 (90)	584 (98)	
	Emergency	86 (10)	13 (2)	
Adjuvant therapy				<0.001
(1463)	**No**	668 (76)	376 (64)	
	Yes	212 (24)	207 (36)	
Tumour characteristics				
Tumor site				<0.001
	Colon	645 (73)	383 (64)	
	Rectum	237 (27)	214 (36)	
T stage				<0.001
	1	59 (7)	96 (16)	
	2	100 (11)	77 (13)	
	3	479 (54)	324 (54)	
	4	244 (28)	100 (17)	
N stage				0.831
	0	549 (62)	367 (62)	
	1	245 (28)	169 (28)	
	2	88 (10)	60 (10)	
TNM stage				0.016
	I	135 (15)	153 (26)	
	II	414 (47)	213 (36)	
	III	333 (38)	231 (39)	
Venous invasion				<0.001
(1473)	**No**	430 (49)	161 (27)	
	Yes	452 (51)	430 (73)	
Less than 12 lymph nodes retrieved				0.396
(1475)	**No**	644 (73)	421 (71)	
	Yes	238 (27)	172 (29)	
Margin involvement				0.016
(1469)	**No**	829 (94)	568 (97)	
	Yes	53 (6)	19 (3)	
Peritoneal involvement				<0.001
	No	663 (75)	565 (95)	
	Yes	219 (25)	32 (5)	
Tumour perforation				0.001
(1477)	**No**	859 (97)	559 (94)	
	Yes	23 (3)	36 (6)	
mGPS				<0.001
	0	543 (61)	502 (84)	
	1	199 (23)	29 (5)	
	2	140 (16)	66 (11)	

Data analysed using χ^2 analysis for linear trend.

Table 3.2 The relationship between modified Glasgow Prognostic Score and clinicopathological characteristics of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Dokkyo Medical University

		Glasgow Royal Infirmary				Dokkyo Medical University			
		mGPS=0	**mGPS=1**	**mGPS=2**		**mGPS=0**	**mGPS=1**	**mGPS=2**	
Clinicopathological Characteristics (*n* when data missing)		***n*=519 (%)**	***n*=166 (%)**	***n*=111 (%)**	***P***	***n*=497 (%)**	***n*=28 (%)**	***n*=59 (%)**	
Host characteristics									
Age					0.007				<0.001
	<65	167 (32)	52 (31)	29 (26)		190 (38)	13 (46)	10 (17)	
	65-74	186 (36)	57 (34)	25 (23)		154 (31)	8 (29)	12 (20)	
	>75	166 (32)	57 (34)	57 (51)		153 (31)	7 (25)	37 (63)	
Sex					0.105				0.322
	Female	227 (44)	83 (50)	56 (50)		195 (39)	4 (14)	22 (37)	
	Male	292 (56)	83 (50)	55 (55)		302 (61)	24 (86)	37 (63)	
ASA grade					0.005				0.026
(1080)	**I/II**	199 (59)	62 (51)	33 (43)		415 (89)	26 (93)	42 (78)	
	III/IV	136 (41)	60 (49)	44 (57)		49 (11)	2 (7)	12 (22)	
Adjuvant therapy					0.933				0.184
(1365)	**No**	400 (77)	120 (72)	88 (79)		311 (64)	16 (57)	42 (75)	
	Yes	118 (23)	46 (28)	23 (21)		175 (36)	12 (43)	14 (25)	
Tumour characteristics									
Tumour site					<0.001				0.005
	Colon	348 (67)	124 (75)	92 (83)		306 (62)	17 (61)	48 (81)	
	Rectum	171 (33)	42 (25)	19 (17)		191 (38)	11 (39)	11 (19)	
T stage					<0.001				<0.001
	1	51 (10)	5 (3)	2 (2)		89 (18)	5 (18)	2 (3)	
	2	83 (16)	11 (7)	5 (4)		72 (15)	1 (3)	4 (7)	
	3	292 (56)	93 (56)	63 (57)		266 (53)	14 (50)	37 (63)	
	4	93 (18)	57 (34)	41 (37)		70 (14)	8 (29)	16 (27)	

Data analysed using χ^2 analysis for linear trend.

Table 3.2 (continued) The relationship between modified Glasgow Prognostic Score and clinicopathological characteristics of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Doyyko Medical University

		Glasgow Royal Infirmary				Dokkyo Medical University			
		mGPS=0	**mGPS=1**	**mGPS=2**		**mGPS=0**	**mGPS=1**	**mGPS=2**	
Clinicopathological Characteristics (*n* when data missing)		***n*=519 (%)**	***n*=166 (%)**	***n*=111 (%)**	***P***	***n*=497 (%)**	***n*=28 (%)**	***n*=59 (%)**	***P***
N stage					0.572				0.115
	0	331 (64)	95 (57)	77 (69)		303 (61)	15 (53)	43 (73)	
	1	152 (29)	47 (28)	23 (21)		143 (29)	8 (29)	14 (24)	
	2	36 (7)	24 (15)	11 (10)		50 (10)	5 (18)	2 (3)	
TNM stage					0.011				0.307
	I	16 (22)	13 (8)	6 (5)		143 (29)	5 (18)	5 (9)	
	II	215 (41)	82 (49)	71 (64)		159 (32)	10 (36)	36 (64)	
	III	188 (36)	71 (43)	34 (31)		195 (39)	13 (46)	16 (27)	
Less than 12 lymph nodes retrieved					0.262				0.941
(1376)	**No**	371 (71)	122 (73)	85 (77)		353 (71)	19 (70)	42 (71)	
	Yes	148 (29)	44 (27)	26 (23)		141 (29)	8 (30)	17 (29)	
Venous invasion					0.492				0.382
(1368)	**No**	257 (50)	87 (52)	58 (52)		138 (28)	2 (30)	13 (22)	
	Yes	262 (50)	79 (48)	53 (48)		354 (72)	19 (70)	46 (78)	
Margin involvement					0.051				0.060
(1370)	**No**	499 (96)	150 (90)	104 (94)		478 (98)	24 (89)	55 (95)	
	Yes	20 (4)	16 (10)	7 (6)		11 (2)	3 (11)	3 (5)	
Peritoneal involvement					<0.001				<0.001
	No	434 (84)	114 (69)	77 (69)		483 (97)	25 (89)	47 (80)	
	Yes	85 (16)	52 (31)	34 (31)		14 (3)	3 (11)	12 (20)	
Tumour perforation					<0.001				0.825
(1378)	**No**	517 (99)	160 (96)	107 (96)		472 (95)	24 (86)	57 (97)	
	Yes	2 (1)	6 (4)	4 (4)		23 (5)	4 (14)	2 (3)	

Data analysed using χ^2 analysis for linear trend.

Table 3.3 Comparison between clinicopathological characteristics and the modified Glasgow Prognostic Score of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Dokkyo Medical University

Clinicopathological Characteristics (*n* when data missing)		**GRI**		**DMU**		
		mGPS=0	**mGPS≥1**	**mGPS=0**	**mGPS≥1**	***P***
Host characteristics						
Age						
	<65	167 (67)	81 (33)	190 (89)	23 (11)	<0.001
	65-74	186 (69)	82 (31)	154 (88)	20 (12)	<0.001
	>75	166 (59)	114 (41)	153 (78)	44 (22)	<0.001
Sex						
	Female	227 (62)	139 (38)	195 (88)	26 (12)	<0.001
	Male	292 (68)	138 (32)	302 (83)	61 (17)	<0.001
ASA grade						
	I/ II	199 (68)	95 (32)	415 (86)	68 (14)	<0.001
	III/ IV	136 (57)	104 (43)	49 (78)	14 (22)	0.002
Adjuvant therapy						
	No	400 (66)	208 (34)	311 (84)	58 (16)	<0.001
	Yes	118 (63)	69 (37)	175 (87)	26 (13)	<0.001
Tumour characteristics						
Tumour site						
	Colon	348 (62)	216 (38)	306 (82)	65 (18)	<0.001
	Rectum	171 (74)	61 (26)	191 (90)	22 (10)	<0.001
T stage						
	1	51 (88)	7 (12)	89 (93)	7 (7)	0.319
	2	83 (84)	16 (16)	72 (93)	5 (7)	0.05
	3	292 (65)	156 (35)	266 (84)	51 (16)	<0.001
	4	93 (49)	98 (51)	70 (74)	24 (26)	<0.001
N stage						
	0	331 (66)	172 (34)	303 (84)	58 (16)	<0.001
	1	152 (68)	70 (32)	143 (87)	22 (13)	<0.001
	2	36 (51)	35 (49)	50 (88)	7 (12)	<0.001
TNM stage						
	I	116 (86)	19 (14)	143 (93)	10 (7)	0.034
	II	215 (58)	153 (42)	159 (77)	48 (23)	<0.001
	III	188 (64)	105 (36)	195 (87)	29 (13)	<0.001
Venous invasion						
	No	257 (64)	145 (36)	138 (87)	21 (13)	<0.001
	Yes	262 (67)	132 (33)	354 (84)	65 (16)	<0.001
Less than 12 lymph nodes retrieved	**No**	371 (64)	207 (36)	353 (85)	61 (15)	<0.001
	Yes	148 (68)	70 (32)	141 (85)	25 (15)	<0.001
Margin involvement						
	No	499 (66)	254 (34)	478 (86)	79 (14)	<0.001
	Yes	20 (47)	23 (53)	11 (65)	6 (35)	0.208
Peritoneal involvement						
	No	434 (69)	191 (31)	483 (87)	72 (13)	<0.001
	Yes	85 (50)	86 (50)	14 (48)	15 (52)	0.887
Tumour perforation						
	No	517 (66)	267 (34)	472 (85)	81 (15)	<0.001
	Yes	2 (17)	10 (83)	23 (79)	6 (21)	<0.001

Data analysed using χ^2 analysis for linear trend.

Table 3.3 displays the difference in prevalence of an elevated mGPS after controlling for clinicopathological characteristics. Even after controlling for such factors (except tumour perforation and peritoneal involvement), patients from GRI were more likely to exhibit an elevated mGPS.

Table 3.4 The relationship between modified Glasgow Prognostic Score, TNM stage and five-year overall survival of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Dokkyo Medical University

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS ≥ 1	
GRI	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Stage I	135	80 (4)	116	85 (4)	19	-
Stage II	368	72 (2)	215	75 (3)	153	69 (4) $^{0.016}$
Stage III	293	53 (3) $^{<0.001}$	188	60 (4) $^{0.003}$	105	40 (5) $^{<0.001/0.001}$
Stage I-III	796	66 (2)	519	71 (2)	277	57 (3) $^{<0.001}$
DMU	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Stage I	153	94 (2)	143	95(2)	10	-
Stage II	207	82 (4)	159	84 (4)	48	73 (10) $^{0.057}$
Stage III	224	84 (3) $^{0.110}$	195	86 (3) $^{0.222}$	29	68 (12) $^{0.004/0.643}$
Stage I-III	584	86 (2)	497	88 (2)	87	73 (8) $^{<0.001}$

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying overall survival within each TNM stage (rows) and prognostic value of TNM stage stratifying overall survival within each mGPS group (columns) for patients from GRI and DMU. OS – cancer-specific survival, SE – standard error. Survival not calculated if $n<20$.

Table 3.4 displays the prognostic value of the combination of TNM stage and mGPS in patients from both GRI and DMU undergoing elective resection of stage I-III colorectal cancer. Whereas five-year overall survival of patients from GRI with stage III disease was 53%, mGPS further stratified survival from 60% to 40% ($P<0.001$). Similarly, whereas five-year overall survival of patients from DMU with stage III disease was 84%, mGPS further stratified survival from 86% to 68% ($P=0.004$).

Table 3.5 The relationship between modified Glasgow Prognostic Score, TNM stage and American Society of Anaesthesiologists grade in patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Dokkyo Medical University

	mGPS = 0		mGPS ≥ 1	
GRI	***n***	**ASA I-II/ III-IV (%)**	***n***	**ASA I-II/ III-IV (%)**
Stage I	60	37 (62)/ 23 (38)	13	6 (46)/ 7 (54)
Stage II	143	84 (59)/ 59 (41)	116	54 (47)/ 62 (53)
Stage III	132	78 (59)/ 54 (41)	70	35 (50)/ 35 (50)
DMU	***n***	**ASA I-II/ III-IV (%)**	***n***	**ASA I-II/ III-IV (%)**
Stage I	131	119 (91)/ 12 (9) $^{<0.001}$	9	7 (78)/ 2 (22) $^{0.147}$
Stage II	152	132 (87)/ 20 (13) $^{<0.001}$	44	35 (79)/ 9 (21) $^{<0.001}$
Stage III	181	164 (91)/ 17 (9) $^{<0.001}$	29	26 (90)/ 3 (10) $^{<0.001}$

Data analysed using χ^2 analysis for linear trend. *P*-value compared to patients from GRI with similar TNM stage and mGPS.

Table 3.6 The relationship between modified Glasgow Prognostic Score, T stage and five-year overall survival of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer in Glasgow Royal Infirmary and Dokkyo Medical University

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS ≥ 1	
GRI	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
T1-2	157	79 (4)	134	82 (4)	23	62 (11) [0.020]
T3	448	68 (2)	292	71 (3)	156	62 (4) [0.002]
T4	191	52 (4) [<0.001]	94	58 (5) [0.013]	98	46 (5) [0.029/0.184]
T1-4	796	66 (2)	519	71 (2)	277	57 (3) [<0.001]
DMU	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
T1-2	173	93 (2)	161	95 (2)	12	-
T3	317	85 (3)	266	87 (3)	51	79 (9) [0.172]
T4	94	71 (8) [0.007]	70	76 (9) [0.097]	24	59 (14) [0.019/0.297]
T1-4	584	86 (2)	497	88 (2)	87	73 (8) [<0.001]

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying overall survival within each T stage (rows) and prognostic value of T stage stratifying overall survival within each mGPS group (columns) for patients from GRI and DMU. OS – cancer-specific survival, SE – standard error. Survival not calculated if $n<20$.

Table 3.7 The relationship between modified Glasgow Prognostic Score and five-year overall survival of patients undergoing potentially curative, elective resection of stage III colorectal cancer with adjuvant chemotherapy in Glasgow Royal Infirmary and Dokkyo Medical University

	All mGPS (mGPS = 0-2)		mGPS = 0		mGPS ≥ 1	
GRI	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Adjuvant therapy	130	70 (4)	89	77 (5)	41	55 (8) [0.002]
DMU	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**	***n***	**5-yr OS % (SE)**
Adjuvant therapy	153	82 (4)	137	85 (4)	16	66 (15) [0.028]

Log-rank *P*-value provided for the differential prognostic value of mGPS in stratifying overall survival of patients receiving adjuvant therapy for patients from GRI and DMU. OS – cancer-specific survival, SE – standard error..

4 The tumour stroma percentage as a determinant of disease progression and prognosis in patients with primary operable colorectal cancer

4.1 Introduction

In addition to the intrinsic properties of the tumour cell, there is increasing appreciation of the importance of components of the tumour microenvironment, including tumour necrosis and host local inflammatory responses, as important determinants of oncological outcome (225, 235). Of more recent interest has been the role of the tumour-associated stroma itself as an important determinant of disease progression and survival in a number of solid cancers (422). The stroma, comprised of cancer-associated fibroblasts and supporting extracellular matrix, may facilitate survival and proliferation of neoplastic cells and promote EMT (423), local invasion and metastatic dissemination (424). In addition, in patients with colorectal cancer, characteristics of the stroma may contribute towards chemoresistance to 5-FU-based chemotherapy (425), and increased recurrence following neoadjuvant radiotherapy for rectal cancer (426).

Therefore it is of interest that an increase in the proportion of tumour-associated stroma within the tumour microenvironment has been associated with poorer survival in a number of solid cancers, including breast (427), oesophageal (428) and colon cancer (429-431). Given the above, it may be hypothesised that assessment of the ratio of stroma to tumour, or tumour stroma percentage (TSP), may act as a surrogate for stromal activity and its subsequent effect on disease progression, chemoresistance and oncological outcome.

It is not clear, however, whether the effect of an expanded stroma on survival is independent of other components of the tumour microenvironment, and in particular the host local inflammatory cell infiltrate. Furthermore, the relationship between TSP, host and tumour characteristics remains unknown. Therefore, the aim of the present study was to examine the relationship between the tumour-associated stroma using TSP,

clinicopathological and tumour microenvironment characteristics, and survival of patients undergoing elective, potentially curative resection of colorectal cancer.

4.2 Patients and Methods

Clinicopathological characteristics

Patients were identified from a prospectively collected and maintained database of elective and emergency colorectal cancer resections undertaken in a single surgical unit at GRI. For the purposes of the present study, patients who on the basis of preoperative computed tomography and laparotomy findings were considered to have undergone elective, potentially curative resection of stage I-III colorectal adenocarcinoma between January 1997 and May 2008 were included. Patients with IBD-associated cancer, cancers arising from the vermiform appendix, and those who received neoadjuvant therapy, or who had undergone emergency resection, local resection or resection with palliative intent were excluded. Patients who died within 30 days of surgery were excluded.

Tumours were staged using the fifth edition of the TNM classification (382), with additional data taken from pathological reports issued following resection. Venous invasion was routinely identified using elastica staining as previously described (432). Patient demographics and pre-operative serum CRP and albumin concentrations were collected prospectively, and the systemic inflammatory response was assessed using the mGPS as described in Chapter 2.

Multi-disciplinary team review, indications for adjuvant therapy and patient follow-up have previously been described in Chapter 2. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 1st December 2011 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Assessment of the tumour microenvironment

Assessment of the inflammatory cell infiltrate using the KM grade, tumour necrosis and TSP were all performed using original H&E-stained sections retrieved from the NHS Greater Glasgow & Clyde pathology archive. Sections were selected from areas of the tumour most representative of the deepest point of tumour invasion.

Klintrup-Mäkinen grade

Assessment of the generalised inflammatory cell infiltrate at the invasive margin using the (KM) grade has previously been performed in this cohort (249). Briefly, the density of the generalised inflammatory cell infiltrate at the invasive margin was graded semi-quantitatively using a four-point scale as described in Table 1.7. For the purpose of further analysis, KM grade was subsequently categorised as low grade or high grade.

T-lymphocyte subsets

On the basis of a previous systematic review (225), the adaptive local inflammatory response within the tumour microenvironment was examined using immunohistochemistry for mature ($CD3^+$), cytotoxic ($CD8^+$), memory ($CD45R0^+$) and regulatory ($FOXP3^+$) T-lymphocyte subsets in a cohort of patients as previously described (433). The density of each T-lymphocyte subset within the invasive margin and intraepithelial (cancer cell nests) compartments was graded semi-quantitatively using a four-point scale (absent/ low/ moderate/ high). For the purposes of analysis, T-lymphocyte density was subsequently graded as low (absent/ low) or high (moderate/ high).

Tumour necrosis

Assessment of tumour necrosis has previously been performed in this cohort using methodology described by Pollheimer and colleagues (235, 434). Briefly, necrosis was semi-quantitatively graded as absent, focal (<10% of tumour area), moderate (10–30% of

tumour area) or extensive (> 30% per cent of tumour area). For the purpose of analysis, tumours were subsequently graded as either low grade (absent/ focal) or high grade (moderate/ extensive).

Tumour stroma percentage

Assessment of TSP was performed using methodology previously described by Mesker and colleagues (429). Tumour sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 optical magnification and visualisation was carried out using Slidepath Digital Image Hub, version 4.0.1, (Slidepath, Leica Biosystems, Milton Keynes, UK). At x5 magnification, an area representative of the tumour invasive margin was selected. Using x10 magnification, a single field was examined, ensuring that tumour cells were present at all four sides of the image. The area of stroma was calculated as a percentage (to the nearest 5%) of the visible field. Areas of necrosis or mucin were excluded from the field for purposes of analysis, and in tumours with a heterogeneous appearance, the area with the highest tumour stromal volume was measured, consistent with the method described by Mesker. Where multiple sections were available, each section was scored and an average value calculated for each tumour. All tumours were scored by a single investigator blinded to clinicopathological data and clinical outcomes (JHP), with co-scoring of 35 patients performed by another investigator (CSDR) to ensure consistency.

Statistical analysis

To identify a threshold for subsequent analysis, patients were split into quartiles on the basis of TSP and survival analysed between each group using Kaplan-Meier log rank (Mantel-Cox) pairwise comparisons. This was subsequently used to categorise patients as low TSP or high TSP. Subsequent analysis was performed to examine the relationship between TSP group and survival using Kaplan-Meier log rank analysis to calculate five-

year survival (SE). Univariate Cox proportional hazards regression was used to calculate HRs and 95% CIs. To identify clinicopathological characteristics independently associated with survival, variables with *P*-value <0.1 were then entered in to a multivariable model using a backwards conditional method. For the purposes of survival analysis, only the KM grade was entered into the multivariate model as a measure of the local inflammatory cell infiltrate as this has previously been shown to have similar prognostic value to assessment of T-lymphocyte subsets (433). The relationship between TSP and other clinicopathological characteristics was examined using the χ^2 test for linear trend. A *P*-value <0.05 was considered statistically significant. All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA).

4.3 Results

Three hundred and thirty-one patients who underwent elective resection of Stage I-III colorectal cancer were included. Clinicopathological characteristics are displayed in Table 4.1. Data on adjuvant therapy and systemic inflammatory responses was missing for one patient. Two thirds of patients were older than 65 years at time of surgery and 52% were male. Over two thirds of patients (70%) underwent colonic resection, with pathological confirmation of stage I disease in 25 patients (8%), stage II in 184 patients (56%) and Stage III disease in 122 patients (37%). Eighty-two patients (25%) received adjuvant chemotherapy; one patient with stage I disease, 22 patients with stage II disease and 59 patients with stage III disease received adjuvant chemotherapy.

The median number of slides scored per patients was 2 (range 1-8). Tumour stroma percentage ranged from 5% to 95%. The interobserver intraclass correlation coefficient was 0.783 for assessment of TSP (>0.7 is considered good). On univariate Cox proportional regression, an incremental increase in TSP was associated with reduced cancer-specific survival (HR 1.02, 95%CI 1.01-1.03, P<0.001). When split into quartiles (Figure 4.1), the survival of patients in the first, second and third quartiles did not differ significantly, however the survival of patients in the fourth quartile (TSP>50%) was significantly worse (quartile 1 (TSP 5%-22%) P=0.001; quartile 2 (TSP 23%-35%) P=0.021; quartile 3 (TSP 36%-50%) P=0.018). As such, patients were classified as low TSP (TSP≤50%) and high TSP (>50%). The interobserver intraclass correlation coefficient for TSP group was 0.813, indicating excellent concordance. Examples of low TSP and high TSP tumours are displayed in Figure 4.2.

The relationship between TSP and clinicopathological characteristics is displayed in Table 4.1. Tumour stroma percentage was not associated with age, sex, or mGPS, however patients with a high TSP were more likely to receive adjuvant chemotherapy (P<0.01). A

high TSP was associated with the presence of adverse pathological characteristics; TSP was associated with increasing T stage ($P<0.05$), N stage, TNM stage (both $P<0.01$), margin involvement and peritoneal involvement (both $P<0.05$). In addition, patients with a high TSP tumour showed a trend towards increased presence of venous invasion ($P=0.066$). There was no relationship between TSP and tumour location, differentiation or tumour perforation.

The relationship between TSP and characteristics of the tumour microenvironment is displayed in Table 4.2. A high TSP was inversely associated with the presence of tumour necrosis and associated with the presence of an infiltrative invasive margin (both $P\leq0.001$). TSP was inversely associated with the density of $CD3^+$ and $CD8^+$ T-cells within the cancer cell nests ($P<0.01$ and $P<0.05$ respectively) but not $CD45R0^+$ or $FOXP3^+$ T-cells. Tumour stroma percentage showed a trend towards an inverse association with KM grade ($P=0.069$), but was not associated with the density of any T-lymphocyte subset at the invasive margin.

The median follow-up of survivors was 107 months (range 44-179 months) and five-year cancer-specific survival was 75%. There were 95 cancer-associated deaths and 66 non-cancer deaths. The relationship between TSP and cancer-specific survival of patients undergoing resection of stage I-III colorectal cancer is displayed in Figure 4.3. A high TSP was significantly associated with poorer five-year cancer-specific survival (81% (3) vs.64% (6), $P<0.001$).

The relationship between TSP, clinicopathological and tumour microenvironment characteristics and cancer-specific survival is shown in Table 4.3. On univariate Cox regression survival analysis, high TSP was associated with shorter cancer-specific survival ($P<0.001$). On multivariate analysis, high TSP was associated with reduced cancer-

specific survival (HR 1.84, 95% CI 1.17-2.92, P=0.009), independent of age (P<0.05), mGPS, N stage (both P<0.001), venous invasion (P<0.05) and K-M grade (P=0.001).

The relationship between TSP and cancer-specific survival of patients undergoing elective resection of node negative colorectal cancer is displayed in Figure 4.3. A high TSP showed a trend towards poorer five-year cancer-specific survival (87% (3) vs. 80% (7), P=0.069). On multivariate survival analysis (Table 4.4), a high TSP was independently associated with reduced cancer-specific survival (HR 2.14, 95% CI 1.01-4.54, p=0.048), independent of mGPS and K-M score (both P<0.05).

The relationship between TSP and cancer-specific survival of 82 patients receiving adjuvant chemotherapy following resection was subsequently examined (Figure 4.3). A high TSP was associated with poorer five-year cancer-specific survival following adjuvant chemotherapy (83% (5) vs. 55% (9), P=0.009). On multivariate analysis (Table 4.5), a high TSP was associated with reduced cancer-specific survival (HR 2.83, CI 1.23-6.53, P=0.015), independent of mGPS, venous invasion, tumour perforation and K-M score (all P<0.05).

4.4 Discussion

In the present study of patients undergoing elective, potentially curative resection of Stage I-III colorectal cancer without neoadjuvant therapy, a high TSP was associated with reduced cancer-specific survival, independent of clinicopathological and tumour microenvironment characteristics. Furthermore, the present threshold of a TSP of 50% is consistent with, and externally validates, previous reports by Mesker and colleagues (429, 430). This simple, rapid assessment of the tumour-associated stroma, using routine pathological specimens, may improve risk stratification of patients undergoing curative resection of colorectal cancer.

Despite being associated with increasing T stage, TSP was inversely associated with tumour necrosis. The basis of this observation was not clear, however stromal expansion may obviate the development of tumour necrosis through increased angiogenesis (435) and resistance to tissue hypoxia (436). Furthermore the tumour-associated stroma may reciprocate in tumour cell metabolism by facilitating the recycling of products of anaerobic metabolism for further use by tumour cells (437). Moreover, the inverse association between TSP and necrosis may also explain the lack of any perceived effect of TSP on the host systemic inflammatory response, as necrosis has been shown to promote systemic inflammation through interleukin-6 and other circulating pro-inflammatory cytokines (438).

The present results suggest that an expanded tumour-associated stroma may influence disease progression through a direct effect on tumour growth and invasive capabilities. Indeed, the presence of a tumour-supporting stroma may overcome barriers to sustained tumour growth and invasion, such as a lack of suitable energy substrate and build-up of metabolic waste (437), tissue hypoxia (436) and host-tissue integrity (424). It was of interest that the proportion of T1-2 and T3 tumours with a high TSP was similar (21% and

19% respectively), whereas 38% of T4 tumours had a high TSP. Therefore, although a high TSP may be identified in earlier stage tumours, expansion of the stromal compartment may predominantly be a characteristic of more locally advanced, aggressive disease, perhaps facilitating tumour budding and host tissue infiltration (439).

Indeed, the present study found an association between TSP and the presence of an infiltrative invasive margin. Furthermore, lymph node metastases and venous invasion were also more likely in patients with a high TSP. Consistent with this, the tumour-associated stroma has previously been shown to facilitate EMT (424, 440) and tumour cell migration into normal tissue at the host-tumour interface, characteristic of an infiltrative invasive margin (439). Similarly, the presence of an immature stroma and a high density of stromal myofibroblasts have both been associated with tumour budding (237, 441). Therefore, the present findings further support a pertinent role for the tumour-associated stroma in facilitating tumour cell de-differentiation and dissemination.

Although the interrelationships between the tumour-associated stroma, tumour microenvironment and gross pathological characteristics are likely complex, TSP remained independently associated with cancer-specific survival in patients undergoing elective, potentially curative resection of colorectal cancer. Furthermore, alongside host local and systemic inflammatory responses, TSP was more strongly associated with reduced cancer-specific survival than pathological characteristics currently used to identify high-risk, node negative disease (172). Indeed, the present results further confirm the importance of both tumour-based factors, such as the tumour microenvironment, and host factors, such as the systemic inflammatory response, in determining oncological outcome.

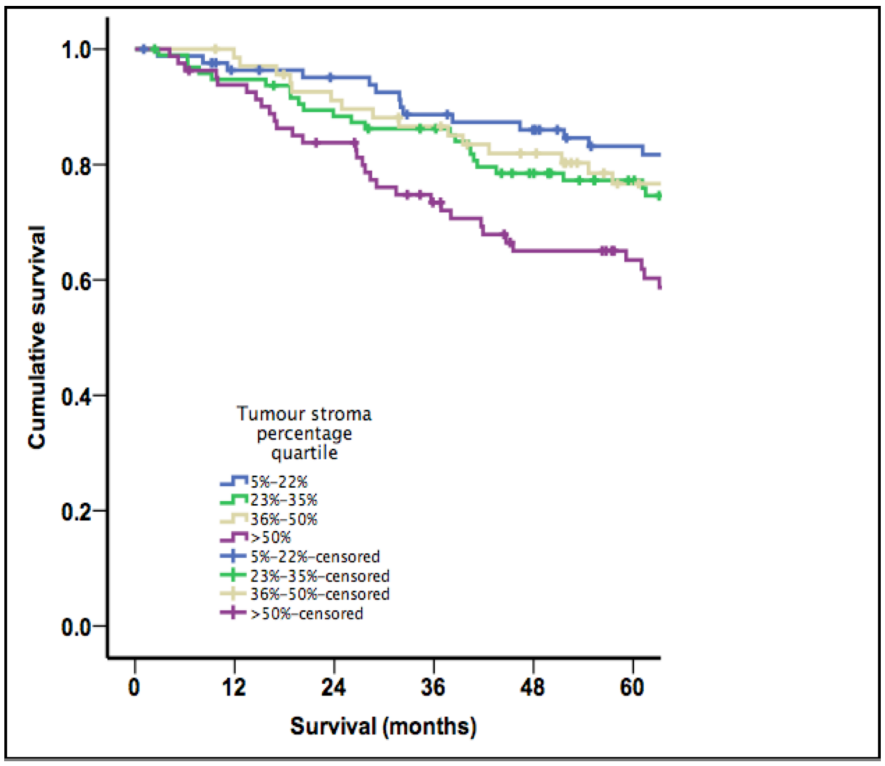
Consistent with previous reports of the role of the tumour-associated stroma as a determinant of chemoresistance (425), survival was significantly shorter in patients undergoing adjuvant therapy for high TSP tumours. In addition to identifying high risk patients, TSP may also select patients less likely to benefit from standard adjuvant therapy and who should be considered for additional adjunctive treatment, potentially targeted at the stroma itself (442). Indeed, given the potential role of the tumour-associated stroma in promoting angiogenesis (435), TSP may well be a biomarker of response to antiangiogenic therapies. However, such agents are not currently licensed for use in patients with non-metastatic colorectal cancer in the UK. Furthermore, relatively few patients in the present study received adjuvant chemotherapy, with less than 50% of patients with Stage III undergoing adjuvant treatment. Therefore, the role of TSP as a prognostic and predictive biomarker remains to be investigated in a larger cohort of patients undergoing adjuvant chemotherapy for high-risk colorectal cancer.

Despite recognition of the importance of the tumour-associated stroma in cancer progression, its relationship with other components of the tumour microenvironment has yet to be fully characterised. In the present study, the presence of a high TSP appeared to preclude infiltration of cancer cell nests by mature ($CD3^+$) and cytotoxic ($CD8^+$) T-lymphocytes. Furthermore, although not reaching statistical significance, TSP displayed an inverse association with the density of the peritumoural inflammatory cell infiltrate as measured by K-M grade but not by T-lymphocyte subsets. Indeed, it has previously been proposed that the tumour-associated stroma may prevent effective tumour infiltration by adaptive immune cells (236, 237, 443). Of interest however, the effect of TSP on survival in the present study remained independent of local inflammatory responses, suggesting that the tumour-associated stroma may influence survival through a number of mechanisms rather than just through a direct effect on adaptive, T-lymphocyte-mediated immunity. Furthermore, although the T-lymphocyte markers examined in the present study were

chosen on the basis of a recent systematic review confirming their relevance in colorectal cancer (225), the relationship between TSP and other cellular components of both the adaptive and innate local inflammatory responses remains to be examined. Indeed, the tumour-associated stroma may promote the development of a pro-tumour rather than anti-tumour immune infiltrate (444). Therefore, further characterisation of the inflammatory infiltrate and its association with the tumour stroma is warranted.

The present study is limited by the small number of patients with stage I disease (25 patients). As such, it was not possible to examine the effect of TSP on clinicopathological characteristics and survival separately in patients with stage I and stage II colorectal cancer, and therefore gain further insight into the natural history and development of the tumour-associated stroma. Despite this limitation, the present study provides comprehensive assessment of the associations between TSP and the tumour microenvironment and, in a cohort of patients with mature survival data, further confirms the prognostic relevance of assessment of the tumour microenvironment in patients undergoing resection.

In summary, the present study further confirms the importance of the tumour microenvironment, and in particular the tumour-associated stroma, in determining oncological outcome in patients with colorectal cancer. Due to its relatively simple assessment, TSP may be readily incorporated into routine clinical pathological reporting to improve risk stratification following potentially curative resection of stage I-III colorectal cancer.



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
5%-22%	84	77	74	68	64	57
23%-35%	96	90	83	79	68	59
36%-50%	69	67	61	57	51	41
>52%	81	75	66	54	45	40

Figure 4.1 The relationship between tumour stroma percentage quartile and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer (log-rank $P<0.001$)

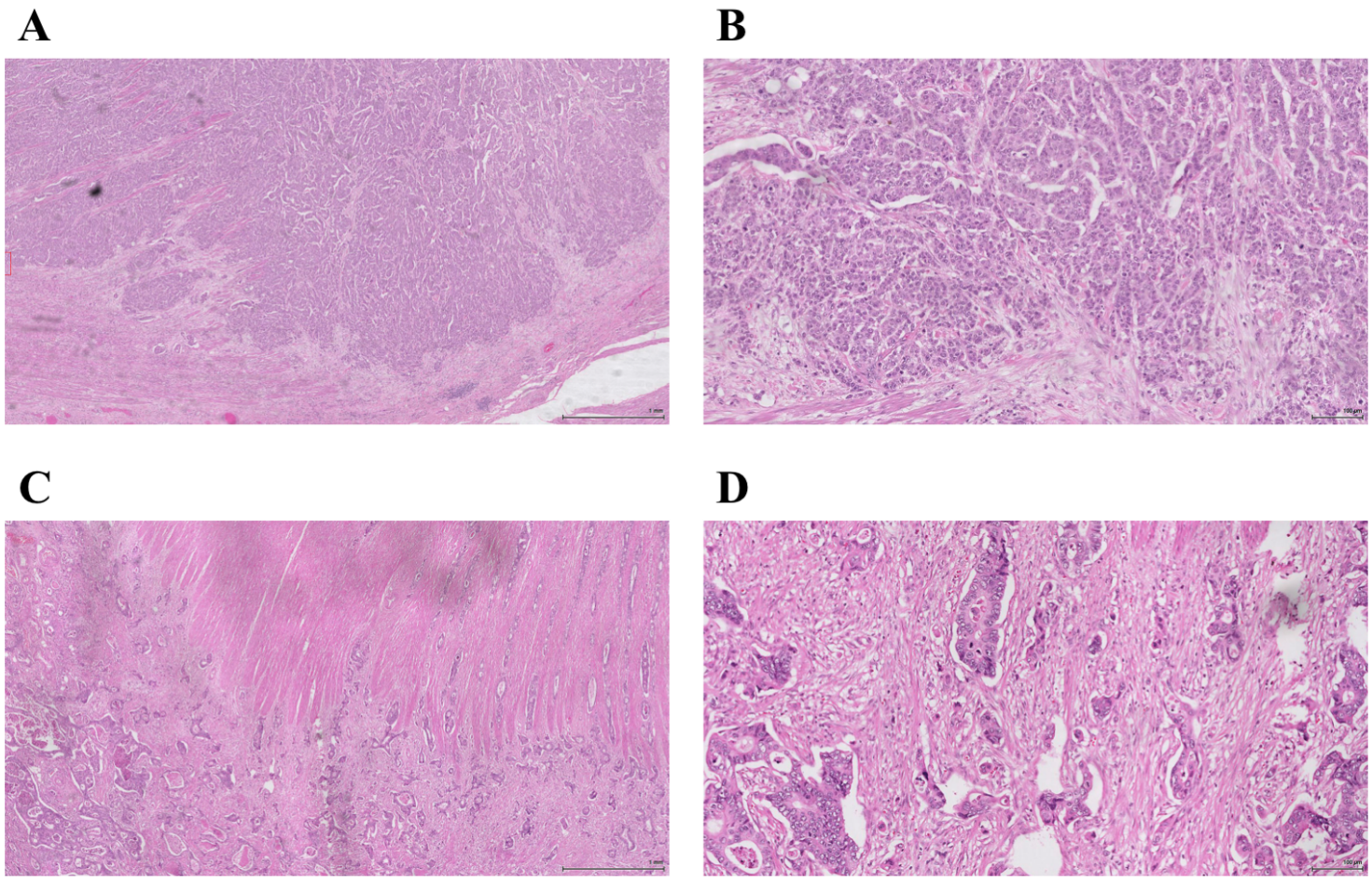
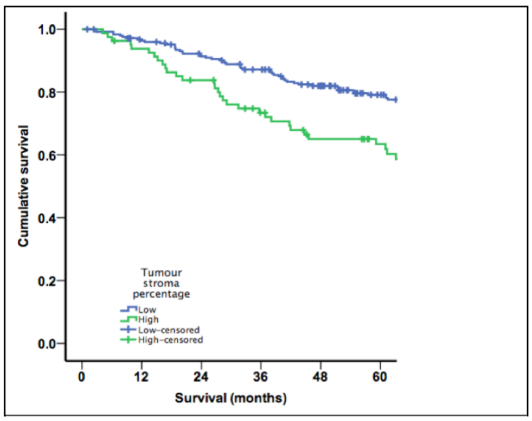


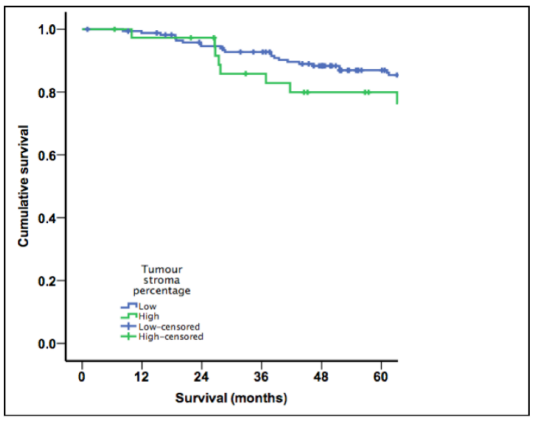
Figure 4.2 Tumour stroma percentage of patients with colorectal cancer as assessed using haematoxylin and eosin-stained sections of the invasive margin. **(A)** and **(B)** an example of low tumour stroma percentage at x20 and x100 magnification, and **(C)** and **(D)** an example of high tumour stroma percentage at x20 and x100 magnification

A



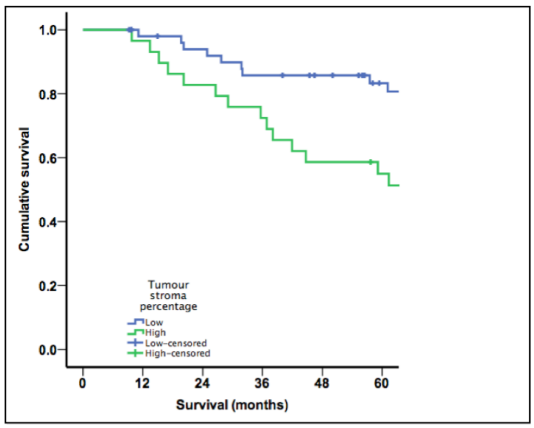
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	250	235	219	205	184	157
High TSP	81	75	66	54	45	40

B



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	171	167	156	150	135	116
High TSP	38	36	35	29	25	23

C



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	53	49	46	42	39	32
High TSP	29	28	24	21	17	15

Figure 4.3 The relationship between tumour stroma percentage and cancer-specific survival of patients undergoing elective, potentially curative resection in **(A)** stage I-III colorectal cancer (P<0.001), **(B)** node negative (stage I-II) colorectal cancer (P=0.069), and **(C)** patients receiving adjuvant chemotherapy (log-rank P=0.009). All P-values calculated using log-rank analysis

Table 4.1 The relationship between tumour stroma percentage and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		All	Low TSP	High TSP	
Clinicopathological Characteristics (*n* when data missing)		***n*=331 (%)**	***n*=250 (%)**	***n*=81 (%)**	***P***
Host characteristics					
Age					0.197
	<65	112 (34)	82 (33)	30 (37)	
	65-74	110 (33)	80 (32)	30 (37)	
	>75	109 (33)	88 (35)	21 (26)	
Sex					0.188
	Female	160 (48)	126 (50)	34 (42)	
	Male	171 (52)	124 (50)	47 (58)	
Modified Glasgow Prognostic					0.177
Score	**0**	194 (59)	143 (57)	51 (64)	
(330)	**1**	90 (27)	67 (27)	23 (28)	
	2	46 (14)	39 (16)	7 (9)	
Adjuvant therapy					0.009
(330)	**No**	248 (75)	196 (79)	52 (64)	
	Yes	82 (25)	53 (21)	29 (36)	
Tumour characteristics					
Tumour site					0.293
	Colon	232 (70)	179 (72)	53 (65)	
	Rectum	99 (30)	71 (28)	28 (35)	
T stage					0.027
	1-2	33 (10)	26 (11)	7 (9)	
	3	208 (63)	168 (67)	40 (49)	
	4	90 (27)	56 (22)	34 (42)	
N stage					0.002
	0	209 (63)	171 (69)	38 (47)	
	1	95 (279	61 (24)	34 (42)	
	2	27 (8)	18 (7)	9 (11)	
TNM stage					0.002
	I	25 (8)	20 (8)	5 (6)	
	II	184 (56)	151 (60)	33 (41)	
	III	122 (37)	79 (32)	43 (53)	
Tumour differentiation					0.564
	Mod/well	292 (88)	222 (89)	70 (86)	
	Poor	39 (12)	28 (11)	11 (14)	
Venous invasion					0.066
	No	216 (65)	170 (68)	46 (57)	
	Yes	115 (35)	80 (32)	35 (43)	
Margin involvement					0.011
	No	310 (94)	239 (96)	71 (88)	
	Yes	22 (6)	11 (4)	10 (12)	
Peritoneal involvement					0.019
	No	249 (75)	196 (78)	53 (65)	
	Yes	82 (25)	54 (22)	28 (35)	
Tumour perforation					0.527
	No	324 (98)	244 (98)	80 (99)	
	Yes	7 (2)	6 (2)	1 (1)	

Data analysed using χ^2 analysis for linear trend.

Table 4.2 The relationship between tumour stroma percentage and components of the tumour microenvironment of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Low TSP	High TSP	
Tumour microenvironment characteristics (*n* when data missing)		***n*=244 (%)**	***n*=87 (%)**	***P***
Tumour necrosis				0.001
(297)	**Low grade**	115 (51)	54 (74)	
	High grade	109 (49)	19 (26)	
Character of margin				<0.001
(312)	**Expansile**	152 (64)	26 (34)	
	Infiltrative	84 (36)	50 (66)	
Klintrup-Mäkinen grade				0.069
(307)	**High grade**	84 (36)	19 (25)	
	Low grade	147 (64)	57 (75)	
CD3$^+$ margin density				0.944
(249)	**High**	80 (57)	28 (44)	
	Low	80 (43)	36 (56)	
CD3$^+$ cancer cell nest density				0.003
(259)	**High**	76 (39)	12 (19)	
	Low	119 (61)	52 (81)	
CD8$^+$ margin density				0.771
(226)	**High**	70 (42)	24 (40)	
	Low	96 (58)	36 (60)	
CD8$^+$ cancer cell nest density				0.046
(226)	**High**	53 (32)	11 (18)	
	Low	113 (68)	49 (82)	
CD45R0$^+$ margin density				0.781
(222)	**High**	77 (47)	26 (45)	
	Low	87 (53)	32 (55)	
CD45R0$^+$ cancer cell nest density	**High**	51 (31)	12 (21)	0.138
(223)	**Low**	114 (69)	46 (79)	
FOXP3$^+$ margin density				0.569
(219)	**High**	68 (42)	22 (38)	
	Low	93 (58)	36 (62)	
FOXP3$^+$ cancer cell nest density				0.237
(219)	**High**	84 (52)	25 (43)	
	Low	77 (48)	33 (57)	

Data analysed using χ^2 analysis for linear trend.

Table 4.3 The relationship between clinicopathological characteristics, components of the tumour microenvironment and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.38 (1.07-1.77)	0.013	1.42 (1.08-1.85)	0.011
Sex (Female/ male)	0.81 (0.54-1.20)	0.291	-	-
mGPS (0/ 1/ 2)	1.75 (1.35-2.27)	<0.001	1.73 (1.29-2.31)	<0.001
Adjuvant therapy (No/ yes)	1.15 (0.73-1.80)	0.548	-	-
Tumour site (Colon/ rectum)	1.18 (0.77-1.81)	0.456	-	-
T stage (1-2/ 3/ 4)	1.43 (1.07-1.92)	0.016	-	0.814
N stage (0/ 1/ 2)	2.19 (1.67-2.88)	<0.001	1.75 (1.29-2.38)	<0.001
Tumour differentiation (Mod-well/ poor)	1.59 (0.90-2.80)	0.110	-	-
Venous invasion (No/ yes)	2.37 (1.58-3.55)	<0.001	1.78 (1.13-2.78)	0.012
Margin involvement (No/ yes)	2.88 (1.54-5.42)	0.001	-	0.580
Peritoneal involvement (No/ yes)	1.86 (1.22-2.84)	0.004	-	0.148
Tumour perforation (No/ yes)	2.69 (0.85-8.55)	0.094	-	0.061
Character of margin (Expansile/ infiltrative)	1.69 (1.11-2.56)	0.014	-	0.883
Tumour necrosis (Low grade/ high grade)	1.42 (0.92-2.19)	0.112	-	-
Klintrup-Mäkinen grade (Low grade/ high grade)	0.32 (0.18-0.57)	<0.001	0.37 (0.21-0.66)	0.001
Tumour stroma percentage (≤50%/ >50%)	2.10 (1.38-3.19)	<0.001	1.84 (1.17-2.92)	0.009

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 4.4 The relationship between clinicopathological characteristics, components of the tumour microenvironment and cancer-specific survival of patients undergoing elective, potentially curative resection of node negative colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.41 (0.95-2.08)	0.089	-	0.087
Sex (Female/ male)	0.97 (0.52-1.80)	0.912	-	-
mGPS (0/ 1/ 2)	1.61 (1.08-2.38)	0.018	1.71 (1.11-2.64)	0.016
Adjuvant therapy (No/ yes)	1.00 (0.36-2.82)	0.996	-	-
Tumour site (Colon/ rectum)	1.38 (0.71-2.69)	0.337	-	-
T stage (1-2/ 3/ 4)	0.92 (0.64-1.32)	0.639	-	-
Tumour differentiation (Mod-well/ poor)	0.96 (0.29-3.10)	0.939	-	-
Venous invasion (No/ yes)	1.96 (1.03-3.74)	0.041	-	0.079
Margin involvement (No/ yes)	1.55 (0.37-6.44)	0.547	-	-
Peritoneal involvement (No/ yes)	1.32 (0.65-2.71)	0.443	-	-
Tumour perforation (No/ yes)	1.82 (0.25-13.43)	0.557	-	-
Character of margin (Expansile/ infiltrative)	1.50 (0.78-2.89)	0.225	-	-
Tumour necrosis (Low grade/ high grade)	1.11 (0.56-2.18)	0.770	-	-
Klintrup-Mäkinen grade (Low grade/ high grade)	0.39 (0.17-0.89)	0.025	0.41 (0.18-0.93)	0.034
Tumour stroma percentage (≤50%/ >50%)	1.88 (0.94-3.78)	0.074	2.14 (1.01-4.54)	0.048

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 4.5 The relationship between clinicopathological characteristics, components of the tumour microenvironment and cancer-specific survival of patients receiving adjuvant chemotherapy following elective, potentially curative resection of colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.16 (0.66-2.02)	0.613	-	-
Sex (Female/ male)	0.8 (0.38-1.81)	0.645		
mGPS (0/ 1/ 2)	1.99 (1.19-3.32)	0.009	1.95 (1.11-3.43)	0.020
Tumour site (Colon/ rectum)	1.06 (0.47-2.37)	0.895	-	-
T stage (1-2/ 3/ 4)	1.55 (0.81-2.96)	0.181	-	-
N stage (0/ 1/ 2)	1.49 (0.80-2.77)	0.210	-	-
Tumour differentiation (Mod-well/ poor)	1.14 (0.39-3.30)	0.814	-	-
Venous invasion (No/ yes)	3.01 (1.31-6.96)	0.010	3.31 (1.33-8.24)	0.010
Margin involvement (No/ yes)	4.77 (1.98-11.49)	0.001	-	0.195
Peritoneal involvement (No/ yes)	2.13 (0.98-4.64)	0.056	-	0.392
Tumour perforation (No/ yes)	5.82 (1.33-25.44)	0.019	6.95 (1.16-41.51)	0.034
Character of margin (Expansile/ infiltrative)	0.85 (0.39-1.84)	0.684	-	-
Tumour necrosis (Low grade/ high grade)	1.19 (0.51-2.76)	0.682	-	-
Klintrup-Mäkinen grade (Low grade/ high grade)	0.17 (0.04-0.70)	0.015	0.21 (0.05-0.88)	0.033
Tumour stroma percentage (≤50%/ >50%)	2.71 (1.25-5.91)	0.012	2.83 (1.23-6.53	0.015

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

5 Mismatch repair status in patients with colorectal cancer: association with phenotypic features of the tumour and the host

5.1 Introduction

In Chapters 2 and 4, assessment of the local and systemic environment of the tumour was shown to determine the prognosis of patients undergoing potentially curative resection of stage I-III colorectal cancer. In particular, systemic and local inflammatory responses, in addition to TSP, held prognostic value independent of conventional, TNM-based staging. However, the characteristics of the tumour that may determine these components of the local and systemic environment remain to be elicited.

One such tumour characteristic is loss of MMR protein activity. Approximately 15-18% of tumours arise through genomic instability as a result of loss of MMR competency; whereas 2% of MMR deficient (dMMR) tumours occur through inherited germline mutations, the remaining 13-15% account for sporadic cases of colorectal cancer, often as a result of hypermethylation-induced silencing of the *hMLH1* promoter region (16). Tumours arising through dMMR activity accumulate mutations at an exponential rate, particularly within repeating microsatellite regions, and are characterised by the presence of MSI as well as distinct phenotypic characteristics, such as proximal tumour location and poor or mucinous differentiation (17-19). Furthermore, dMMR status is associated with improved survival, particularly in patients with Stage II/III colorectal cancer (151, 198, 420).

In addition to such characteristics, dMMR colorectal cancer is associated with a typical tumour microenvironment phenotype; in particular, the presence of a high density of tumour infiltrating lymphocytes has been consistently reported (17, 19, 20, 445). Furthermore, the presence of a low proportion of tumour-associated stroma has similarly been associated with dMMR status (430). Indeed, the improved prognosis attributed to

dMMR status may not be entirely independent of such favourable characteristics within the tumour microenvironment (200, 201, 430).

Despite extensive characterisation of the tumour microenvironment, it is of interest that the relationship between MMR status and the systemic environment remains to be fully defined. Given the favourable prognosis associated with dMMR status, it would be expected that patients with tumours arising through this pathway would be less likely to exhibit evidence of a cancer-associated systemic inflammatory response at diagnosis. Therefore, the aim of the present study was to characterise the relationships between MMR status, the local and systemic environment and survival of patients undergoing elective, potentially curative resection of colorectal cancer.

5.2 Patients and Methods

Clinicopathological characteristics

Patients were identified from a prospectively collected and maintained database of elective and emergency colorectal cancer resections undertaken in a single surgical unit at GRI. For the purposes of the present chapter, patients who had undergone elective, primary resection of stage I-III colorectal adenocarcinoma between January 1997 and May 2007, and who had tumour tissue included in a previously constructed colorectal cancer tissue microarray (TMA) were included (446). In addition to exclusion criteria described in Chapter 4, patients with a known or suspected hereditary colorectal cancer syndrome were excluded. Clinicopathological staging, multi-disciplinary team review, indications for adjuvant chemotherapy and clinical follow-up have previously been described in Chapter 2.

Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Assessment of the tumour microenvironment

The KM grade and TSP were both assessed using routine H&E-stained sections of the deepest point of tumour invasion as previously described in Chapter 4. The KM grade was categorised as low grade or high grade, and TSP was categorised as low (≤50%) or high (>50%).

Full sections of the invasive margin were stained for mature ($CD3^+$), cytotoxic ($CD8^+$), memory ($CD45R0^+$) and regulatory ($FOXP3^+$) T-lymphocyte subsets as previously

described in Chapter 4. The density of each T-lymphocyte subset within the invasive margin or cancer cell nests was graded as either high or low density.

Assessment of the systemic inflammatory responses

Pre-operative serum C-reactive protein (CRP), albumin and the mGPS were measured as previously described in Chapter 2. In addition, the differential white cell count measured at the same time was also recorded prospectively. The NLR was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. On the basis of previously derived thresholds, neutrophil count>$7.5x10^9$, platelet count>$400x10^9$/L and NLR>5were considered elevated (385, 447). The neutrophil:platelet score (NPS) was calculated as described by Watt and colleagues (448): patients with a normal platelet count and neutrophil count were allocated a score of 0, either an elevated neutrophil count or platelet count a score of 1, and those with both an elevated neutrophil and platelet count a score of 2.

Assessment of mismatch repair status

Previously constructed TMAs, comprising of four 0.6mm cores of formalin-fixed paraffin-embedded cancer tissue per patient, were utilised to assess MMR status (446). TMA slides were placed in a ThermoFisher pH 9 PT module solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) at room temperature. Slides were then heated in the PT module to a temperature of 96^oC for 20 minutes and allowed to cool. Using the ThermoFisher autostainer, slides were incubated in peroxidase block for 5 minutes and rinsed with TBS before incubating in UV protein blocker for 5 minutes and rinsing once again with TBS solution. Slides were incubated in primary antibody for 20 minutes at a concentration of 1:100 for MLH1 and MSH6 and 1:50 for MSH2 and PMS2 (product codes: M3640, M3646, M3639 and M3647 respectively; Dako UK Ltd, Cambridgeshire, UK). Following this incubation period, slides were rinsed with TBS and Quanto Amplifier (Thermo Fisher

Scientific Inc.) was applied to slides for 10 minutes followed by a further wash with TBS. Quanto Polymer was then added for 10 minutes followed by a TBS wash. DAB Quanto substrate was then added for 5 minutes, slides washed in TBS, counterstained in haematoxylin, blued in Scotts' tap water, and dehydrated through a series of graded alcohols before applying cover slips with distrene, plasticizer and xylene (DPX) mounting medium.

The expression of MMR proteins was established by a single observer (AGP) blinded to clinical outcomes using UK NEQAS scoring guidelines (22). Appendix and normal colon were used as positive controls and positive staining within intratumoural immune cells served as an internal positive control. An example of positive and negative staining for MLH1 protein expression is displayed in Figure 5.1. One observer blinded to clinical outcome (JHP) scored 10% of cores. Expression was reported as MMR proficient (tumour cell nuclear expression with positive immune cell expression) or MMR deficient (absent tumour nuclear expression with normal immune cell expression). The use of multiple TMA cores per patient has been shown to be comparable to the use of full sections, even in the presence of known intratumoural heterogeneity of protein expression (449). In the present study, four cores were examined per patient for each MMR protein; TMA assessment of MLH1 and MSH2 using three cores per patient has previously been shown to be comparable to use of full section analysis (450).

Statistical analysis

The relationship between MMR status, clinicopathological characteristics and characteristics of the local and systemic environment was examined using the χ^2 method for linear trend for categorical variables and Mann-Whitney U test for continuous variables. The relationship between MMR status, local and systemic environment characteristics associated with MMR status and survival was examined by Kaplan-Meier

log-rank survival analysis and Cox proportional hazards regression using a multivariate backwards conditional model to calculate HRs and 95% CIs. A *P*-value ≤0.05 was considered statistically significant. All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). The West of Scotland Research Ethics Committee approved the study and tissue for analysis of MMR status was obtained from the NHS Greater Glasgow & Clyde Tissue Biorepository.

5.3 Results

A total of 228 patients who underwent elective, potentially curative resection of stage I-III colorectal cancer were included. Clinicopathological characteristics of the cohort are displayed in Table 5.1. Almost two thirds of patients were older than 65 at time of surgery and 53% were male. Pathological assessment confirmed Stage I disease in 16 patients (7%), stage II disease in 111 patients (49%) and stage III disease in 101 patients (44%). Sixty-six patients (29%) received adjuvant therapy; 1 patient with stage I disease, 15 patients with stage II disease and 50 patients with stage III disease received adjuvant therapy. Mismatch repair deficiency was identified in 35 patients (15%); the frequency of aberrant MMR protein expression in patients with dMMR colorectal cancer is displayed in Table 5.2.

The relationship between mismatch repair status and clinicopathological characteristics

The relationship between MMR status and clinicopathological characteristics is displayed in Table 5.1. Patients with dMMR colorectal cancer were more likely to have a colonic primary and exhibit poor tumour differentiation (both $P<0.05$). In addition, although not associated with T stage, dMMR status was associated with an increased rate of peritoneal involvement ($P<0.05$). Detection of dMMR did not differ with year of diagnosis ($P=0.290$). Furthermore, the age of patients with dMMR colorectal cancer did not differ significantly from those with MMR competent cancer ($P=0.707$). As such, it is unlikely that a significant proportion of included patients had Lynch syndrome-associated cancer.

The relationship between mismatch repair status and the tumour microenvironment

The relationship between MMR status and the tumour microenvironment is displayed in Table 5.3. Patients with dMMR colorectal cancer had an increased density of $CD3^+$ ($P<0.01$), $CD45R0^+$ ($P<0.05$) and $CD8^+$ ($P=0.071$) T-lymphocytes within the cancer cell

nests. Although not reaching statistical significance, patients with dMMR colorectal cancer were less likely to have a high TSP (15% vs. 28%, *P*=0.118). The density of $FOXP3^+$ T-lymphocytes within the cancer cell nests, density of T-lymphocytes at the invasive margin, nor the KM grade, showed significant association with MMR status.

The relationship between mismatch repair status and systemic inflammatory responses

The relationship between MMR status and systemic inflammatory responses is displayed in Figure 5.2 and Table 5.4. Patients with dMMR colorectal cancer had a higher median pre-operative CRP (P<0.001) and neutrophil count (P<0.05), and showed a trend towards a higher median platelet count (P=0.091). Serum albumin concentrations and circulating lymphocyte count did not differ with MMR status. Patients with dMMR colorectal cancer were more likely to have a neutrophil count>7.5x109/L (P<0.01) and platelet count >400x109/L (P<0.05). In addition, both the mGPS and NPS were more likely to be elevated in patients with dMMR colorectal cancer (both P<0.01).

The relationship between mismatch repair status, the local and systemic environment and survival

The relationship between MMR status, characteristics of the local and systemic inflammatory responses significantly associated with MMR status, and cancer-specific survival was subsequently examined (Table 5.5). The median follow-up of survivors was 143 months (range 87-206 months) with 66 cancer-specific deaths and five-year cancer-specific survival of 76%. On multivariate survival analysis, dMMR was not significantly associated with cancer-specific survival (*P*=0.790), whereas the density of $CD3^+$ T-lymphocytes within the cancer cell nests (*P*<0.001), mGPS (*P*<0.01) and NPS (*P*<0.05) were independently associated with survival. When analysis was restricted to patients with stage II/III disease only, cancer cell nest $CD3^+$ T-lymphocyte density (*P*<0.001), mGPS

and NPS (both $P<0.05$) remained associated with survival independent of MMR status ($P=0.833$).

As cancer cell nest density of CD3+ T-lymphocytes, mGPS and NPS were all associated with survival independent of MMR status, the relationship between these characteristics and cancer-specific survival of patients with MMR competent colorectal cancer was subsequently examined (Figure 5.3). Five-year cancer specific survival was stratified from 94% to 67% by cancer cell nest CD3+ T-lymphocyte density (P<0.001), from 83% to 46% by mGPS (P=0.002) and from 78% to 60% by NPS (P=0.054).

5.4 Discussion

The results of the present study describe the distinct tumour and host phenotypic characteristics associated with MMR deficiency in patients undergoing elective, potentially curative resection of colorectal cancer. Patients with dMMR colorectal cancer were more likely to have a high density of T-lymphocytes within the tumour microenvironment and evidence of an elevated host systemic inflammatory response as evidenced by components of the differential white cell count and serum acute phase proteins. Furthermore, these characteristics were associated with cancer-specific survival independent of MMR status. Taken together with the previous literature (201, 254, 430, 451), this provides further evidence that the prognostic benefit associated with dMMR colorectal cancer is not necessarily independent of such characteristics.

Patients with dMMR colorectal cancer were more likely to have a high density of intratumoural $CD3^+$, $CD8^+$ and $CD45R0^+$ T-lymphocytes, however dMMR status did not appear to influence $FOXP3^+$ T-regulatory lymphocyte density. Furthermore, it was of interest that the inflammatory cell infiltrate at the invasive margin, as measured by either T-lymphocyte density or KM grade, did not differ with MMR status. Given that the KM grade is reflective of components of both adaptive and innate local immune responses (452), the present study would favour an association between dMMR status and development of a primarily co-ordinated, adaptive intratumoural immune response. Indeed, this is consistent with recent work addressing the nature of the immune microenvironment in patients with dMMR colorectal cancer (445, 453). De Smedt and colleagues recently reported that MSI-associated colon cancers primarily elicited an intratumoural, lymphocytic inflammatory response with little change in the peritumoural generalised inflammatory infiltrate (445). Secondly, Maby and co-workers reported that an increased burden of MSI-associated frameshift mutations predominantly favoured tumour infiltration by $CD8^+$ T-lymphocytes but not $FOXP3^+$ T-lymphocytes (453). Taken

together with these prior studies, the present results further support the role of dMMR in promoting tumour infiltration by a co-ordinated, adaptive, anti-tumour lymphocytic response (295).

An unexpected finding was an association between dMMR status and the presence of an elevated systemic inflammatory response. In particular, dMMR status was associated with an elevated CRP, neutrophil count and platelet count, as well prognostic scores derived from these markers. Of interest however, and consistent with recent work by Pine and colleagues (454), neither circulating lymphocyte count nor NLR were associated with MMR status. Although Pine and colleagues hypothesised that the peritumoural lymphocytosis associated with dMMR colorectal cancer may translate into an increase in circulating lymphocyte count, the results of the present study more closely reflect our understanding of the nature of the cancer-associated systemic inflammatory response. Whereas the presence of a conspicuous inflammatory cell infiltrate within the tumour microenvironment reflects the presence of an adaptive, anti-tumour immune response, it is increasingly appreciated that an elevated systemic inflammatory response primarily reflects up-regulation of mediators of innate immunity, which in turn promote tumour progression and dissemination (398). As such, it would be expected that any association between tumour characteristics and the systemic inflammatory response would be reflected by changes in markers of innate immunity, such as circulating CRP concentrations and neutrophil and platelet counts.

The mechanism underlying an association between systemic inflammation and MMR status is not clear. Although dMMR tumours may be more likely to express an "inflammatory response"-type gene signature (455), another possible explanation is that the presence of a chronic systemic inflammatory response may predispose patients to sporadic development of dMMR tumours (16, 456). For example, the pro-inflammatory cytokine IL-6 has previously been implicated in the initiation of mismatch repair defects in

colon cancer cell lines (457), and a similar relationship between systemic inflammation and MMR status has been observed in patients with gynaecological malignancies (456). Furthermore, despite dMMR tumours eliciting a profound anti-tumour lymphocytic immune response, it has recently been shown that this is counterbalanced by up-regulation of multiple immune checkpoints (295). Indeed, whether the systemic inflammatory response reflects underlying immune checkpoint activation, or may be indicative of an activated common upstream precursor, such as the JAK/STAT3 pathway, would be of considerable interest (458).

Characterisation of host local and systemic inflammatory responses was a stronger predictor of survival than assessment of MMR status on multivariate survival analysis, and also showed prognostic value in patients with MMR competent colorectal cancer, consistent with previous reports (201, 451, 452, 459, 460). Furthermore, a considerable proportion of patients with MMR competent colorectal cancer had a high density of intraepithelial T-lymphocytes. Given that assessment of MMR status alone would have failed to identify these patients, it is clear that combined assessment of host local and systemic inflammatory responses, in conjunction with MMR status and standard pathological staging, could potentially lead to better risk stratification than each of these measures used individually.

The results of the present chapter are perhaps limited by the use of immunohistochemistry to identify loss of MMR activity rather than genetic sequencing for microsatellite instability. Not all MSI pathway tumours will be identifiable by loss of MMR proteins (461), however immunohistochemistry-based detection of MLH1 and MSH2 has an acceptable sensitivity and specificity for microsatellite instability screening (462), and this is further improved by the use of the additional markers PMS2 and MSH6 as utilised in the present study (461). In addition, previous studies have found that immunohistochemistry-based assessment of MMR status utilising TMA sections is comparable to full-section

analysis (450, 463). Whereas prior studies have recommended the use of three cores per tumour (450), the present analysis was performed using four cores for each protein. Furthermore, although the use of older, archival tissue can influence the results of immunohistochemistry, there was no difference in the frequency of detection of MMR deficiency with year of surgery, suggesting that this was not an issue in the present study. Finally, manual semi-quantitative assessment of the local inflammatory cell infiltrate was presently employed rather than automated assessment. However, this has been shown to have excellent inter-operator agreement (433), and correlates strongly with automated digital assessment (445, 464).

In summary, the results of the present study further highlight the complexities of the relationship between the local and systemic tumour environment and MMR status in patients with colorectal cancer. Furthermore, these results further confirm the importance of the local and systemic environment, in addition to the intrinsic properties of tumour cells, in determining outcome of patients with colorectal cancer.

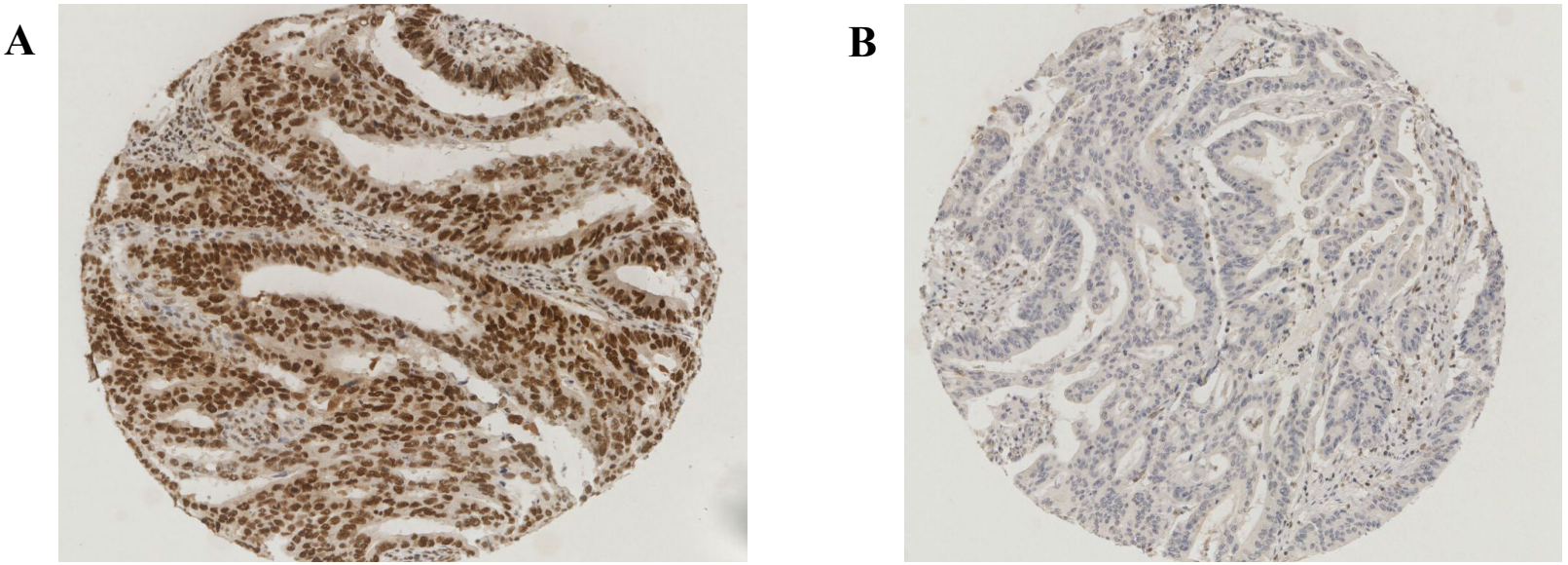


Figure 5.1 Example of mismatch repair protein expression of patients with colorectal cancer (x200 magnification). **(A)** positive MLH1 expression within tumour epithelium, and **(B)** lack of MLH1 expression within tumour epithelium. Positive staining of intratumoural lymphocytes provides a positive internal control

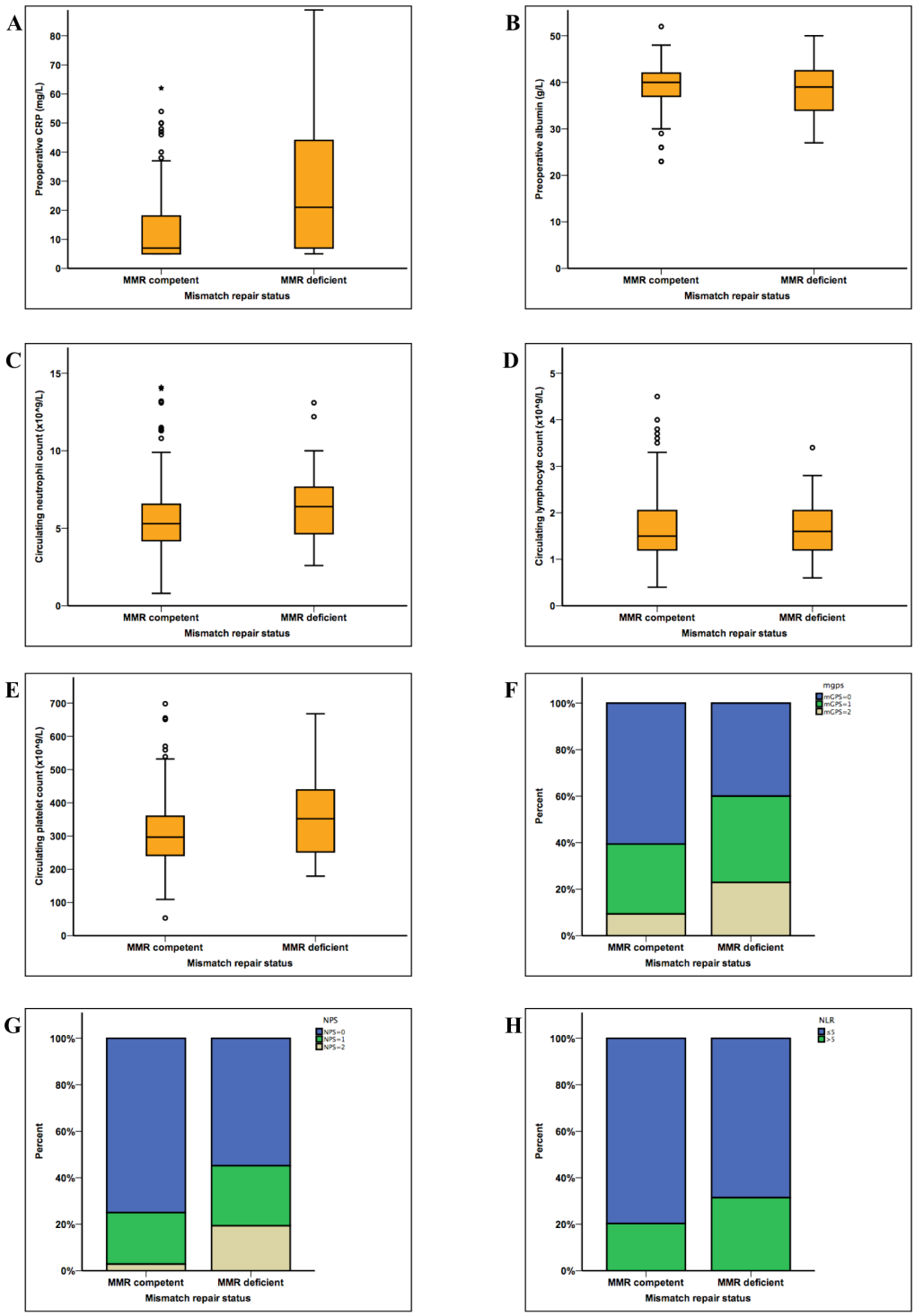
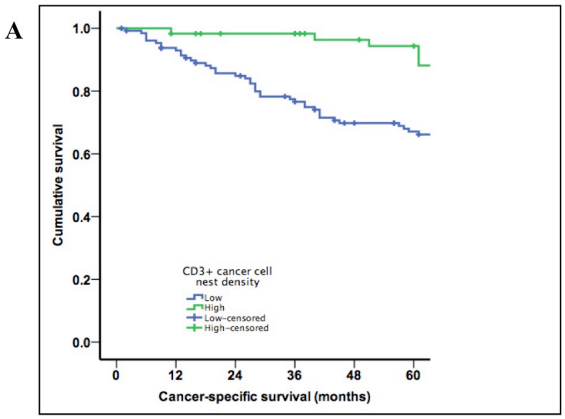
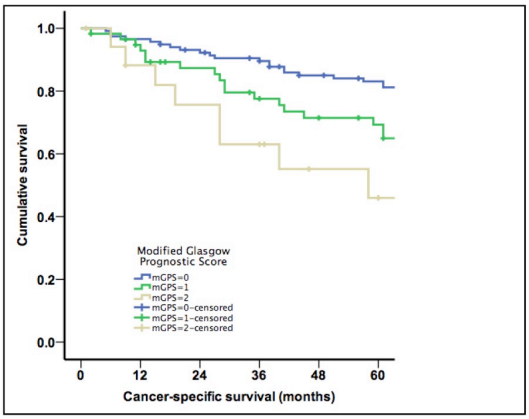


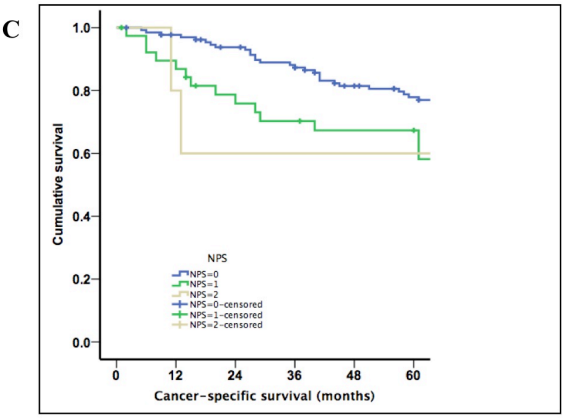
Figure 5.2 The relationship between mismatch repair status and host systemic inflammatory responses in patients undergoing elective, potentially curative resection of stage I-III colorectal cancer. **(A)** serum C-reactive protein (P<0.001), **(B)** serum albumin (P=0.258), **(C)** circulating neutrophil count (P=0.032), **(D)** circulating lymphocyte count (P=0.669), **(E)** circulating platelet count (P=0.091), **(F)** modified Glasgow Prognostic Score (P=0.007), **(G)** neutrophil: platelet score (P=0.001), and **(H)** neutrophil: lymphocyte ratio (P=0.145). All P-values calculated using Mann-Whitney U test



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
High density	59	57	54	54	49	47
Low density	130	117	105	93	80	74



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
mGPS 0	117	112	106	101	91	87
mGPS 1	58	52	45	39	35	32
mGPS 2	18	14	12	10	6	5



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
NPS 0	132	125	117	109	95	87
NPS 1	39	34	28	25	23	23
NPS 2	5	4	3	3	3	3

Figure 5.3 The relationship between tumour and host characteristics associated with survival independent of mismatch repair status and cancer-specific survival of patients undergoing elective, potentially curative resection of mismatch repair competent, stage I-III colorectal cancer. **(A)** cancer cell nest $CD3^+$ T-lymphocyte density (P<0.001), **(B)** modified Glasgow Prognostic Score (P=0.002), and **(C)** neutrophil: platelet score (P=0.054). All P-values calculated using log-rank analysis

Table 5.1 The relationship between mismatch repair status and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		All	MMR competent	MMR deficient	
Clinicopathological Characteristics		***n*=228 (%)**	***n*=193 (%)**	***n*=35 (%)**	***P***
Host characteristics					
Age					0.707
	<65	83 (36)	71 (37)	12 (34)	
	65-74	73 (32)	62 (32)	11 (32)	
	>75	72 (32)	60 (31)	12 (34)	
Sex					0.832
	Female	108 (47)	92 (48)	16 (46)	
	Male	120 (53)	101 (52)	19 (54)	
Diagnosis Year					0.290
	1997-2002	142 (62)	123 (64)	19 (54)	
	2003-2007	86 (38)	70 (36)	16 (46)	
Adjuvant therapy					0.389
	No	162 (71)	135 (70)	27 (77)	
	Yes	66 (29)	58 (30)	8 (23)	
Tumour characteristics					
Tumour location					0.024
	Colon	151 (66)	122 (63)	29 (83)	
	Rectum	77 (34)	71 (37)	6 (17)	
T stage					0.365
	1-2	25 (11)	21 (11)	4 (11)	
	3	141 (62)	124 (64)	17 (49)	
	4	62 (27)	48 (25)	14 (40)	
N stage					0.160
	0	127 (55)	105 (54)	22 (63)	
	1	77 (34)	65 (34)	12 (34)	
	2	24 (11)	23 (12)	1 (3)	
TNM stage					0.539
	I	16 (7)	14 (7)	2 (6)	
	II	111 (49)	91 (47)	20 (57)	
	III	101 (44)	88 (46)	13 (37)	
Tumour differentiation					0.039
	Mod/well	200 (88)	173 (90)	27 (77)	
	Poor	28 (12)	20 (10)	8 (23)	
Venous invasion					0.381
	No	148 (65)	123 (64)	25 (71)	
	Yes	80 (35)	70 (36)	10 (29)	
Margin involvement					0.997
	No	215 (94)	182 (94)	33 (94)	
	Yes	13 (6)	11 (6)	2 (6)	
Peritoneal involvement					0.029
	No	165 (72)	145 (75)	20 (57)	
	Yes	63 (28)	48 (25)	15 (43)	
Tumour perforation					0.337
	No	223 (98)	188 (97)	35 (100)	
	Yes	5 (2)	5 (3)	0 (0)	

Data analysed using χ^2 analysis for linear trend.

Table 5.2 Patterns of aberrant mismatch repair protein expression in patients undergoing elective, potentially curative resection of mismatch repair deficient, stage I-III colorectal cancer

Aberrant protein expression	Number of patients
MLH1/PMS2	17
MSH6/MSH2	8
PMS2	7
MSH6	1
PMS2/MSH6	1
PMS/MSH6/MSH2	1

Table 5.2 displays the observed patterns of MMR deficiency in the present cohort of patients undergoing elective resection of stage I-III colorectal cancer. The high prevalence of aberrant MLH1/PMS2 expression suggests that this cohort is likely to predominantly be comprised of patients with sporadic MMR deficient colorectal cancer.

Table 5.3 The relationship between mismatch repair status and tumour microenvironment of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

Data analysed using χ^2 analysis for linear trend

		All	MMR competent	MMR deficient	
Tumour microenvironment (*n* when data missing)		***n*=228 (%)**	***n*=193 (%)**	***n*=35 (%)**	***P***
Klintrup-Mäkinen grade					0.398
	Low	77 (34)	63 (33)	14 (40)	
	High	151 (66)	130 (67)	21 (60)	
CD3$^+$ margin density					0.867
(215)	**Low**	118 (55)	100 (55)	18 (56)	
	High	97 (45)	83 (45)	14 (44)	
CD3$^+$ cancer cell nest density					0.009
(224)	**Low**	146 (65)	130 (69)	16 (46)	
	High	78 (35)	59 (31)	19 (54)	
CD8$^+$ margin density					0.319
(216)	**Low**	127 (59)	105 (57)	22 (67)	
	High	89 (41)	78 (43)	11 (33)	
CD8$^+$ cancer cell nest density					0.071
(222)	**Low**	161 (72)	140 (75)	21 (60)	
	High	61 (28)	47 (25)	14 (40)	
CD45R0$^+$ margin density					0.564
(217)	**Low**	112 (52)	96 (53)	16 (47)	
	High	105 (48)	87 (47)	18 (53)	
CD45R0$^+$ cancer cell nest density					0.015
(224)	**Low**	160 (71)	141 (75)	19 (54)	
	High	64 (29)	48 (25)	16 (46)	
FOXP3$^+$ margin density					0.413
(216)	**Low**	126 (58)	104 (57)	22 (65)	
	High	90 (42)	78 (43)	12 (35)	
FOXP3$^+$ cancer cell nest density					0.731
(219)	**Low**	110 (50)	92 (50)	18 (53)	
	High	109 (50)	93 (50)	16 (47)	
Tumour stroma percentage					0.118
(225)	**Low**	166 (74)	138 (72)	28 (85)	
	High	59 (26)	54 (28)	5 (15)	

Table 5.4 The relationship between mismatch repair status and systemic inflammatory responses of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		All	MMR competent	MMR deficient	
Systemic environment (*n* when data missing)x		***n*=228 (%)**	***n*=193 (%)**	***n*=35 (%)**	***P***
CRP (mg/L)	**Median (IQR)**	8 (6-20)	7 (5-18)	21 (7-48)	<0.001
Albumin (g/L)	**Median (IQR)**	40 (36-42)	40 (37-42)	39 (34-43)	0.258
Modified Glasgow Prognostic Score					0.007
	0	131 (58)	117 (61)	14 (40)	
	1	71 (31)	58 (30)	13 (37)	
	2	26 (11)	18 (9)	8 (23)	
Neutrophil count ($x10^9$/L)	**Median (IQR)**	5.4 (4.3-6.7)	5.3 (4.2-6.6)	6.4 (4.6-7.7)	0.032
Lymphocyte count ($x10^9$/L)	**Median (IQR)**	1.5 (1.2-2.1)	1.5 (1.2-2.1)	1.6 (1.2-2.1)	0.891
Platelet count ($x10^9$/L)	**Median (IQR)**	300 (245-369)	296 (242-360)	352 (251-441)	0.091
Neutrophil count (227)					0.004
	≤7.5x10^9/L	192 (85)	168 (87)	24 (69)	
	>7.5x10^9/L	35 (15)	24 (13)	11 (31)	
Lymphocyte count (227)					0.669
	≤4x10^9/L	226 (99)	191 (99)	35 (100)	
	>4x10^9/L	1 (0)	1 (1)	0 (0)	
Platelet count (207)					0.018
	≤400x10^9/L	171 (83)	150 (85)	21 (68)	
	>400x10^9/L	36 (17)	26 (15)	10 (32)	
Neutrophil:lymphocyte ratio (227)					0.145
	≤5	177 (78)	153 (80)	24 (69)	
	>5	50 (22)	39 (20)	11 (31)	
Neutrophil:platelet score (207)					0.001
	0	149 (72)	132 (75)	17 (55)	
	1	47 (23)	39 (22)	8 (26)	
	2	11 (5)	5 (3)	6 (19)	

Categorical data analysed using χ^2 analysis for linear trend. Continuous data analysed using Mann-Whitney U test. IQR – interquartile range.

Table 5.5 The relationship between local and systemic environment characteristics associated with mismatch repair status and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival	
All patients (*n*=228)	**Multivariate analysis**	***P***
$CD3^+$ cancer cell nest density (Low/ high)	0.28 (0.14-0.57)	<0.001
$CD45R0^+$ cancer cell nest density (Low/ high)	0.69 (0.28-1.72)	0.430
Modified Glasgow Prognostic Score (0/ 1/ 2)	1.59 (1.12-2.27)	0.010
Neutrophil: platelet score (0/ 1/ 2)	1.47 (1.01-2.14)	0.042
Mismatch repair status (Competent/ deficient)	0.69 (031-1.54)	0.367
Stage II/Stage III only (*n*=212)		
$CD3^+$ cancer cell nest density (Low/ high)	0.30 (0.15-0.61)	0.001
$CD45R0^+$ cancer cell nest density (Low/ high)	0.77 (0.30-1.95)	0.578
Modified Glasgow Prognostic Score (0/ 1/ 2)	1.52 (1.06-2.19)	0.023
Neutrophil: platelet score (0/ 1/ 2)	1.46 (1.01-2.13)	0.047
Mismatch repair status (Competent/ deficient)	0.71 (0.32-1.58)	0.399

Data analysed using Cox proportional hazards regression using a backwards conditional method to calculate hazard ratios and 95% confidence intervals.

6 Signal Transduction and Activator of Transcription-3 in patients with colorectal cancer: association with phenotypic features of the tumour and the host

6.1 Introduction

Another potential mechanism linking local and systemic inflammatory responses in patients with colorectal cancer is activation of the JAK/STAT3 pathway by IL-6. Circulating IL-6 is commonly elevated in a number of cancers, including colorectal cancer (438, 465, 466), and is the predominant stimulus for the hepatic synthesis of acute phase proteins, including CRP (261). Cancer-associated fibroblasts and inflammatory cells contribute to high levels of IL-6 within the tumour microenvironment (467, 468), with subsequent tumour cell activation of the soluble IL-6 receptor/ glycoprotein 130 complex (469). Interleukin-6 trans-signalling regulates JAK activity within the tumour cell to promote phosphorylation of the tyrosine 705 residue of STAT3. Phosphorylated STAT3 (pSTAT3) translocates to the nucleus where it is a key transcription factor for numerous T_h2-type cytokines, including IL-6 (466, 467), in turn promoting a pro-tumour, immunosuppressive environment and attenuating host anti-tumour immune responses (218, 468). Indeed, given its role in not only de-regulation of the host anti-tumour immune response, but also in orchestrating numerous pro-oncogenic processes (229, 468, 470), it is not surprising that STAT3 expression and activation has previously been associated with reduced survival in a number of gastrointestinal cancers, including colorectal cancer (471).

Given the above, it can be hypothesised that the host systemic and local inflammatory responses in patients with colorectal cancer may be linked by STAT3. As such, the aim of the present study was to examine the relationship between tumour cell STAT3 expression, the local and systemic environment and survival in a cohort of patients undergoing potentially curative, elective resection of stage I-III colorectal cancer.

6.2 Patients and Methods

Patients who had undergone elective, primary resection of stage I-III colorectal adenocarcinoma without neoadjuvant chemoradiotherapy and who had tumour tissue included in a previously constructed colorectal cancer TMA were included as previously described in Chapter 5. Routine follow-up of patients following surgery has previously been described in Chapter 2. Mismatch repair status was determined as described in Chapter 5. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Assessment of the tumour microenvironment

The KM grade and TSP were both assessed using routine H&E-stained sections of the deepest point of tumour invasion as previously described in Chapter 4; KM grade was categorised as low grade or high grade, and TSP was categorised as low (≤50%) or high (>50%). Full sections of the invasive margin were stained for mature ($CD3^+$), cytotoxic ($CD8^+$), memory ($CD45R0^+$) and regulatory ($FOXP3^+$) T-lymphocyte subsets as previously described in Chapter 4. The density of each T-lymphocyte subset within the invasive margin or cancer cell nests was graded as either high or low density.

Assessment of the systemic inflammatory responses

Pre-operative serum CRP, albumin and the mGPS were measured as previously described in Chapter 2. The NLR and NPS were calculated as described in Chapter 5.

Assessment of STAT3 expression

Immunohistochemical assessment of STAT3 activity was performed using a previously constructed colorectal cancer TMA as described in Chapter 5 (446). In addition to total

STAT3 expression, phosphorylated $STAT3_{Tyr705}$ (pSTAT3) expression was measured as a marker of activation of STAT3 by IL-6/JAK. Sections were dewaxed in xylene before being rehydrated using graded alcohols. Antigen retrieval was performed using a citrate buffer at 96ºC for 20 minutes for STAT3, and using a Tris-EDTA buffer at high pressure in a microwave for 5 minutes for pSTAT3. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 minutes before rinsing in water. Casein and 5% horse serum in TBS were applied for 20 minutes at room temperature as a blocking agent for STAT3 and pSTAT3 respectively. Sections were then incubated overnight at 4ºC with the primary antibody (STAT3: product code 9132, Cell Signaling Technologies; pSTAT3: product code 9131, Cell Signaling Technologies) at a concentration of 1:100 and 1:50 for STAT3 and pSTAT3 respectively before washing in TBS for ten minutes. Envision (Dako) was then added to the sections for 30 minutes at room temperature before washing in TBS for ten minutes. DAB substrate was added for five minutes until colour developed before washing in running water for ten minutes. Slides were counterstained in haematoxylin for 60 seconds and blued with Scotts' tap water before dehydration through graded alcohols. Cover slips were applied using DPX.

Sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualization was carried out using Slidepath Digital Image Hub (Slidepath, Leica Biosystems, Milton Keynes, UK). Assessment of STAT3 and pSTAT3 expression within the cancer cell cytoplasm and nucleus was performed at x20 magnification by a single examiner (JHP) blinded to clinical data using the weighted histoscore (472). To ensure reproducibility of scoring, 15% of tumours were co-scored by a second investigator (JC); the intraclass correlation coefficient was 0.826 and 0.837 respectively. For the purposes of the present study, cytoplasmic localisation of STAT3 expression was considered representative of total STAT3 expression, whereas

nuclear localisation of STAT3 and pSTAT3 expression was considered representative of STAT3 transcriptional activation.

Statistical analysis

Patients were divided into tertiles (low/ moderate/ high) on the basis of cytoplasmic and nuclear STAT3 and pSTAT3 expression. The relationship between clinicopathological characteristics and cytoplasmic and nuclear STAT3 expression was examined using the χ^2 method for linear trend. The relationship between STAT3 expression and five-year cancer-specific survival was examined using Kaplan-Meier log-rank analysis and displayed as percentage surviving (SE). The relationship between STAT3 expression, clinicopathological characteristics and cancer-specific survival was examined using Cox proportional hazards regression; variables with a $P \leq 0.1$ on univariate analysis were entered into a multivariate model using a backwards conditional model to calculate HRs and 95% CIs. A *P*-value ≤0.01 was considered statistically significant for Chi-square analysis of categorical variables to compensate for multiple comparisons, whereas a *P*-value ≤0.05 considered statistically significant for survival analysis. All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). The West of Scotland Research Ethics Committee approved the study and tissue for analysis of STAT3 expression was obtained from the National Health Service Greater Glasgow & Clyde Tissue Biorepository.

6.3 Results

A total of 196 patients who underwent elective, potentially curative resection of stage I-III colorectal cancer were included. Clinicopathological characteristics are displayed in Table 6.1. Almost two thirds of patients were older than 65 at time of surgery and 52% were male. Pathological assessment confirmed Stage I disease in 16 patients (8%), stage II disease in 94 patients (48%) and stage III disease in 86 patients (44%). Fifty-four patients (28%) received adjuvant therapy; 1 patient with stage I disease, 14 patients with stage II disease and 39 patients with stage III disease received adjuvant therapy. Mismatch repair deficiency was identified in 27 patients (14%). Expression of STAT3 was observed in both the cytoplasm and nucleus, whereas pSTAT3 expression was only observed in the nucleus. An example of tumour epithelial expression of STAT3 and pSTAT3 is displayed in Figure 6.1.

Tumour cytoplasmic expression of STAT3 was associated with nuclear expression of STAT3 (Spearman's r=0.363, P<0.001) but not pSTAT3 (r=0.111, P=0.121). Nuclear STAT3 expression showed a trend towards an association with nuclear pSTAT3 expression (r=0.130, P=0.068). Normal, non-cancer epithelium expression of STAT3 was available for 10 patients. Although this precluded meaningful statistical analysis, it was of interest that 7 patients showed similar or higher expression of cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 in normal tissue compared to cancer tissue. The remaining three patients showed heterogeneous expression of each of the studied markers.

The relationship between STAT3 expression and clinicopathological characteristics

The relationship between STAT3 and pSTAT3 expression tertiles and clinicopathological characteristics is displayed in Table 6.1. Cytoplasmic expression of STAT3 was not associated with any clinicopathological characteristics. Although failing to reach statistical significance (P≤0.01), nuclear STAT3 expression showed an inverse association with use

of adjuvant chemotherapy (P=0.038), whereas pSTAT3 expression was associated with younger age (P=0.026) and an increased prevalence of lymph node positive disease (low pSTAT3 expression – 35% vs. high pSTAT3 expression – 52%, P=0.039).

The relationship between STAT3 expression and the tumour microenvironment

The relationship between STAT3 and pSTAT3 expression and components of the tumour microenvironment is displayed in Table 6.2. Cytoplasmic STAT3 expression was inversely associated with the cancer cell nest density of $CD8^+$ and $FOXP3^+$ T-lymphocytes (both P<0.01) and showed a trend towards a similar relationship with $CD3^+$ density (P=0.012) but was not associated with TSP or the local inflammatory cell density at the invasive margin as measured by Klintrup-Mäkinen grade or T-lymphocyte density. Nuclear expression of STAT3 showed no statistically significant association with characteristics of the tumour microenvironment, however a lower density of $CD8^+$ (P=0.039) and $CD3^+$ (P=0.055) T-lymphocytes was observed in patients with high nuclear STAT3 expression. There were no statistically significant associations between nuclear pSTAT3 expression and tumour microenvironment characteristics; patients with high nuclear pSTAT3 expression however were observed to have a lower density of $CD45R0^+$ T-lymphocytes (P=0.037).

When analysis was restricted to patients with MMR competent colorectal cancer, the observed trends between cytoplasmic STAT3 and cancer cell nest density of $CD3^+$ (P=0.061) $CD8^+$ (P<0.05) and $FOXP3^+$ (P<0.01) T-lymphocytes remained. Nuclear STAT3 was no longer associated with $CD8^+$ density within cancer cell nests but was associated with $CD3^+$ density within the invasive margin (P<0.05). Nuclear pSTAT3 expression again showed a non-significant trend towards low cancer cell nest density of $CD45R0^+$ T-lymphocytes. Although the small number of patients limited statistical power, when analysis was restricted to patients with MMR deficient colorectal cancer, the

relationship between cytoplasmic STAT3 expression and cancer cell nest density of $CD3^+$ ($P<0.05$) and $CD8^+$ ($P<0.01$) T-cells, and nuclear STAT3 expression and cancer cell nest density of $CD8^+$ T-cells ($P<0.05$) remained. Nuclear pSTAT3 expression, however, was not associated with T-lymphocyte density of patients with MMR deficient colorectal cancer.

The relationship between STAT3 expression and systemic inflammatory responses

The relationship between STAT3 and pSTAT3 expression and systemic inflammatory responses is displayed in Table 6.3. Cytoplasmic STAT3 expression was associated with the systemic inflammatory response as measured by mGPS; this was predominantly due to an increase in the number of patients with mGPS=2 (high expression – 19% vs. low expression 4%, $P=0.004$). Similarly, nuclear STAT3 expression showed a similar trend in the number of patients with mGPS=2, however this failed to reach statistical significance (18% vs. 8%, $P=0.244$). Neither cytoplasmic nor nuclear STAT3 expression were associated with the systemic inflammatory response as measured by circulating platelets or components of the differential white cell count. Nuclear pSTAT3 expression was not associated with the systemic inflammatory response.

The relationship between STAT3 expression and survival

The median follow-up of survivors was 143 months (range 101-204) with 57 cancer-associated deaths and 64 non-cancer deaths. For the purposes of survival analysis, low and moderate expression of each marker was combined to form one group (low expression). The relationship between cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 and cancer-specific survival is displayed in Figure 6.2 and in Table 6.4. High nuclear STAT3 expression was associated with poorer cancer-specific survival ($P<0.05$). High expression of both cytoplasmic STAT3 expression and nuclear pSTAT3 expression showed a non-significant trend towards poorer survival ($P=0.068$ and $P=0.116$ respectively).

To examine the relationship between expression and activation of STAT3 and survival, the cumulative prognostic value of cytoplasmic STAT3, nuclear STAT3 and nuclear pSTAT3 was examined with respect to five-year cancer-specific survival (Table 6.4). Three models were examined: model 1 (cytoplasmic STAT3/ nuclear STAT3) stratified survival from 81% (low expression of both) to 63% (high expression of both) (P=0.022), model 2 (cytoplasmic STAT3/ nuclear pSTAT3) stratified survival from 81% to 54% (P=0.018), and model 3 (nuclear STAT3/ nuclear pSTAT3) stratified survival from 81% to 62% (P=0.012). When the three models were entered into a multivariate model using a backwards conditional method, only model 3 (nuclear STAT3/ nuclear pSTAT3) remained independently associated with cancer-specific survival (HR 1.63, 95%CI 1.14-2.34 P=0.008, Figure 6.2).

The relationship between combined nuclear STAT3/pSTAT3 expression, tumour characteristics and survival

The relationship between combined assessment of nuclear STAT3/pSTAT3 expression and tumour characteristics was subsequently examined (Table 6.5). Combined nuclear STAT3 and pSTAT3 expression was inversely associated with the density of $CD3^+$ and $CD45R0^+$ T-lymphocytes within the invasive margin and cancer cell nests. Furthermore, increasing nuclear expression of STAT3/ pSTAT3 showed a trend towards a decrease in the density of $CD8^+$ T-lymphocytes within the cancer cell nests (P=0.153) and an increase in TSP (P=0.056). No significant association with the systemic inflammatory response was identified.

Finally, the relationship between combined nuclear STAT3 and pSTAT3 expression and cancer-specific survival was examined on multivariate analysis. As the prognostic value of the KM grade has previously been shown to be similar to assessment of individual T-lymphocyte subsets (433), only KM grade was entered into the multivariate model. On multivariate survival analysis (Table 6.6), combined nuclear STAT3/ pSTAT3 expression

showed a trend towards reduced cancer-specific survival (HR 1.39, 95%CI 0.94-2.06, *P*=0.102). Venous invasion (HR 2.82, 95%CI 1.58-5.04, *P*<0.001), mGPS (HR 1.79, 96%CI 1.18-2.70, *P*=0.006), KM grade (HR 2.23, 95%CI 1.04-4.81, *P*=0.04) and TSP (HR 2.75, 95%CI 1.55-4.89, *P*=0.001) were all independently associated with survival.

6.4 Discussion

In the present study of patients undergoing potentially curative colorectal cancer resection, STAT3 was not strongly associated with clinicopathological characteristics of the tumour but was associated with adverse host inflammatory responses. In particular, increased tumour cell STAT3 expression was associated with down-regulation of the local inflammatory cell infiltrate.

Although in keeping with previous clinical studies of colorectal and pancreatic adenocarcinoma (473, 474), the present study is to our knowledge the first to examine the relationship between tumour STAT3 expression and the density of the local adaptive immune infiltrate as evidenced by T-lymphocytes in the clinical context of patients with gastrointestinal cancer. Whereas previous studies found a decrease in the density of the generalised inflammatory cell infiltrate or tumour-infiltrating lymphocytes using H&E-based assessments (473, 474), the present study utilised immunohistochemistry and found a decrease in the density of tumour-associated T-lymphocyte populations. This would suggest a direct effect of STAT3 activation on adaptive, T-lymphocyte-mediated anti-tumour immunity. Furthermore, the relationship between STAT3 expression and the local inflammatory cell infiltrate would appear to be independent of MMR status.

Although assessment of both cytoplasmic STAT3 expression and combined nuclear STAT3 and pSTAT3 expression were both significantly associated with the density of T-lymphocytes, it was of interest that the KM grade, an assessment of the generalised inflammatory cell infiltrate, did not differ with STAT3 expression. This may reflect the ability of STAT3 to simultaneously suppress anti-tumour immune responses whilst promoting pro-tumour immunity (218, 475). Whereas anti-tumour, adaptive, T_h1-polarised immune responses are down-regulated (476, 477), STAT3-dependent transcription and release of T_h2-type cytokines favours recruitment of tumour-promoting macrophages and

myeloid-derived cells (218). Furthermore, STAT3 activation may additionally favour the differentiation of naïve T-lymphocytes into tumour-promoting lymphocytic subsets (218). Consistent with such a hypothesis, Morikawa and colleagues found that although intratumoural lymphocyte density decreased, the density of the peritumoural inflammatory cell infiltrate increased with increasing STAT3 activity in a cohort of patients with stage I-IV colorectal cancer (473). Furthermore, it has been shown in some tumours, such as ependymomas, that STAT3 immunosuppression is mediated by up-regulation of myeloid-derived cell activity, with a deleterious effect on T-lymphocytic, anti-tumour activity (478). As such, future studies of STAT3 activation in patients with gastrointestinal cancers should also consider the nature and density of local innate immune responses.

Although failing to reach statistical significance, it was of interest that the density of tumour-associated stroma, as measured by TSP, appeared to be associated with both pSTAT3 and combined nuclear STAT3/pSTAT3 activation. Given that an increase in TSP primarily reflects an increased population of cancer-associated fibroblasts within the tumour microenvironment, this would further support the importance of IL-6 secretion by fibroblasts in the activation of the JAK/STAT3 pathway in tumour cells (467, 468). Indeed, the present results suggest that the JAK/STAT3 pathway may be an important mechanism by which the tumour influences the composition of the tumour microenvironment and deregulates host anti-tumour immune responses.

Increased tumour cytoplasmic STAT3 expression was associated with elevated systemic inflammatory responses as measured using the mGPS, a cumulative score based on serum CRP and albumin concentrations. Such routinely measured biomarkers of the systemic inflammatory response represent only “the tip of a far larger iceberg” of cancer-associated systemic inflammation, whereby circulating cytokines, growth factors and myeloid-derived cells promote cancer progression and dissemination (398). One such cytokine, IL-6, is commonly elevated in colorectal cancer (438, 465), and is the main determinant of hepatic

synthesis of CRP and responsible for the acute phase reduction in hepatic albumin synthesis (261). Given the importance of IL-6 as both an activator of the JAK/STAT3 pathway and as an end product of its activation, the present results are not surprising, and suggest that STAT3 may play a role in the systemic inflammatory response in colorectal cancer.

However, although cytoplasmic STAT3 expression was associated with an elevated mGPS, it was not associated with measures of the differential white cell count. This is in keeping with previous work from Guthrie and colleagues, whereby serum IL-6 concentration correlated strongly with the mGPS but not the NLR in patients with colorectal cancer (438). However, other groups have found contradictory results, with a positive association between serum IL-6 concentrations and the NLR in patients with colorectal cancer. This disparity may be explained by differences in the groups studied; whereas the patients in the present analysis, and that of Guthrie and colleagues were undergoing potentially curative resection of stage I-III colorectal cancer, the groups studied by Kantola and Chen included patients with stage I-IV colorectal cancer at varying stages of treatment (465, 479). Therefore, it would appear that at least in patients with non-metastatic colorectal cancer, the effects of the IL-6/JAK/STAT3 pathway on the systemic inflammatory response may not be modulated by an effect on circulating innate and adaptive immune cells.

Of interest, only total cytoplasmic STAT3 expression and not nuclear STAT3/ pSTAT3 activation was associated with the systemic inflammatory response as measured by mGPS. The reason for this is not clear, however may represent the dynamic nature of JAK/STAT3 activation and translocation. Although activation of the IL-6 receptor leads to rapid accumulation of STAT3, mechanistic studies have shown that less than 30% of total cytoplasmic STAT3 translocates to the nucleus on cytokine stimulation (480). Furthermore, STAT3 also exhibits transcription-independent activity within the cytoplasm without nuclear translocation (480, 481). Another plausible hypothesis is that rather than

being directly causative, the presently observed associations between the mGPS and tumour cell STAT3 expression may represent separate down-stream events of a common precursor, such as elevated systemic IL-6 concentrations. Finally, given the lack of a consistent relationship across different measures of the systemic inflammatory response, the present results may simply represent a Type-I statistical error. Indeed, rather than the tumour itself, other end organs, such as liver or skeletal muscle, may be the predominant drivers of the systemic inflammatory response in such patients (482). As such, the present observations should be regarded as hypothesis-generating, and remain to be further investigated by mechanistic and clinical studies.

Consistent with previous reports in patients with gastrointestinal cancers, (471), increased tumour cell STAT3 expression and activitation was associated with reduced survival. The importance of the pleiotropic nature of STAT3 activation is reflected in the fact that combined assessment of total STAT3 and pSTAT3 held greater prognostic value than either measure alone. Whereas the present analysis investigated IL-6/JAK-mediated activation of STAT3 by phosphorylation of tyrosine residue 705, mitogen-activated protein kinase-dependent activation results in phosphorylation of the serine 727 residue, with differing results on transcriptional activity (483). Furthermore, STAT3 may also undergo nuclear import without phosphorylation (484). In addition to its role in mediating host immune responses, STAT3 activation plays an integral role in many key tumour cell pathways, including proliferation, EMT and promotion of cancer cell stemness (485). As such, rather than targeting upstream activation of STAT3, future therapeutic strategies may benefit from targeting STAT3 itself and its subsequent activation.

In the present study, assessment of the local and systemic environment held greater prognostic value than STAT3 and pSTAT3 expression or activation. Rather than being defined by one mechanism such as the JAK/STAT3 pathway, characteristics within the tumour microenvironment and the systemic inflammatory response are multifactorial in

origin. Therefore, it would be expected that such phenotypic characteristics would be of greater prognostic value than a single, contributory pathway. Indeed, it would be of considerable interest to examine and compare similar inflammatory pathways, such as the NF-κB pathway (486, 487), in future studies.

The present study provides further clinical evidence of the potential role of the IL-6/JAK/STAT3 pathway in the amelioration of host anti-tumour immune responses, and raises two interesting points that remain to be investigated. Firstly, it would suggest a role for inhibitors of the IL-6/JAK/STAT3 pathway in restoring anti-tumour immune responses in patients with colorectal cancer (488, 489). Secondly, it would support the hypothesis that routine markers of the systemic inflammatory response, and in particular the mGPS, may act as predictive biomarkers for patients likely to benefit from such targeted therapies (490). In keeping with such a scheme, one recent clinical trial of a JAK inhibitor in patients with metastatic pancreatic cancer found an increase in overall survival only in those patients with an elevated CRP or mGPS (491). Therefore, it is clear that markers of the host inflammatory response should be incorporated into future studies of agents targeting the IL-6/JAK/STAT3 pathway in cancer.

Given the increasing appreciation of distinct molecular subtypes of colorectal cancer (492), the results of the present study are perhaps limited by the lack of molecular characterisation of the tumours studied. Although not associated with MMR status in the present cohort, the relationship between STAT3 and other characteristics, such as *KRAS* and *BRAF* status, would be of interest. However, a previous comprehensive study by Morikawa and colleagues found no association between STAT3, a number of molecular characteristics and survival in a cohort of over 700 patients (473). Furthermore, it has also been suggested that STAT3 may have a role in not only induction of *KRAS* mutated tumours (493), but also in conferring chemoresistance in patients with *KRAS* wild-type

tumours (494). Indeed, this would suggest that STAT3 is independent of such characteristics. A further limitation is the relatively small sample size, precluding meaningful subgroup analysis. Analysis was restricted to a previously constructed TMA, and only patients who had complete staining for both STAT3 and pSTAT3 were included. However, post-hoc power calculation shows that the present study has adequate power to examine the relationship between STAT3 and the local and systemic environment. For example, post-hoc analysis suggests that the present study holds 84% power to determine a difference in cancer cell nest $CD8^+$ T-lymphocyte density between those with low and high cytoplasmic STAT3 expression. Finally, although immunohistochemistry is useful for assessment of protein expression and localisation, other techniques may be more useful for examining STAT3 activation. For instance, the use of gel shift assays would yield more information regarding the transcriptional activity of STAT3 (495).

In conclusion, the results of the present study suggest a relationship between tumour cell STAT3 expression and activation and local and systemic inflammatory responses, and may be one potential mechanism whereby the tumour promotes an environment amenable to tumour growth and dissemination. Further studies are required to confirm such a relationship, and whether therapeutic targeting of the IL-6/JAK/STAT3 may be utilised in the treatment of patients with colorectal cancer and elevated systemic inflammatory responses.

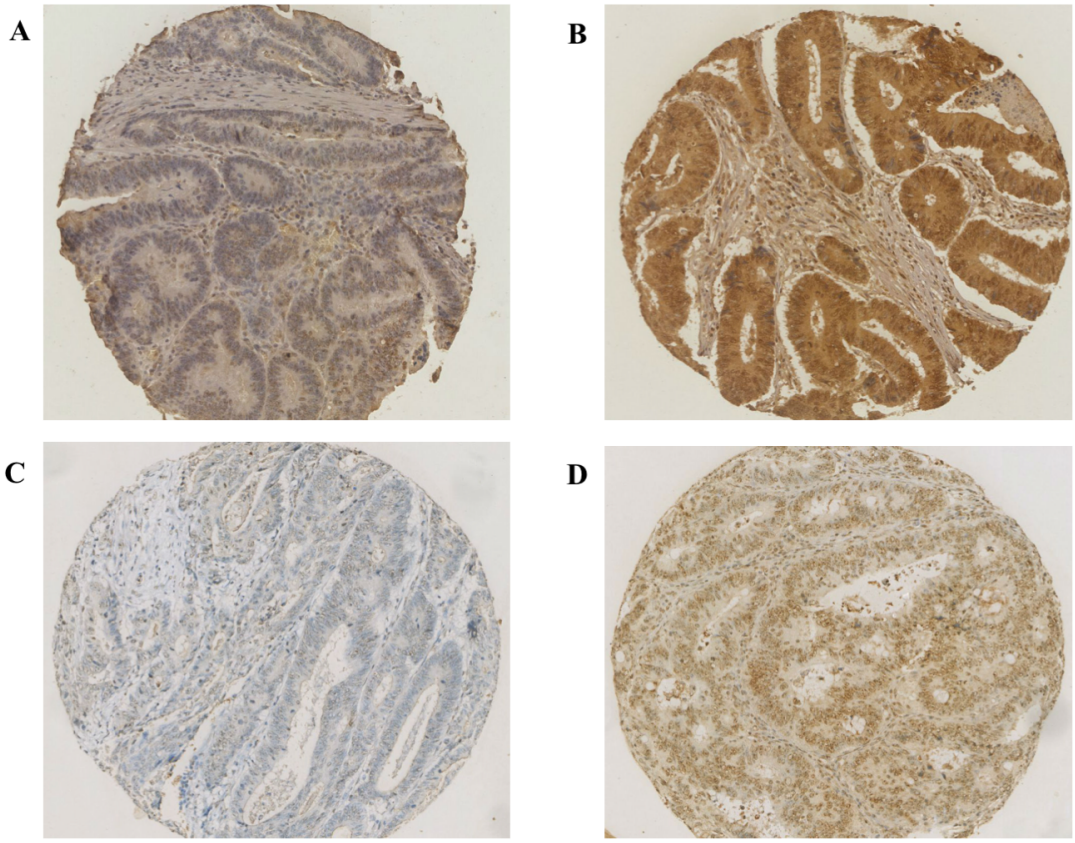
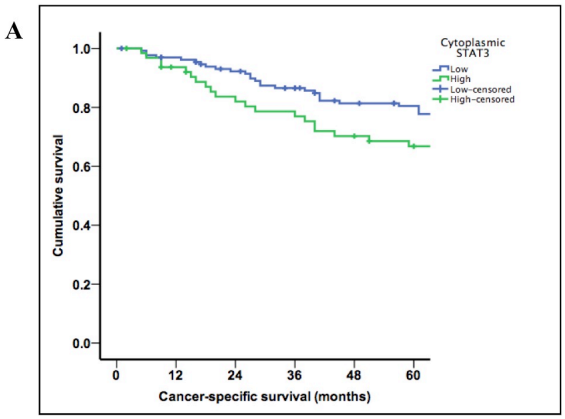
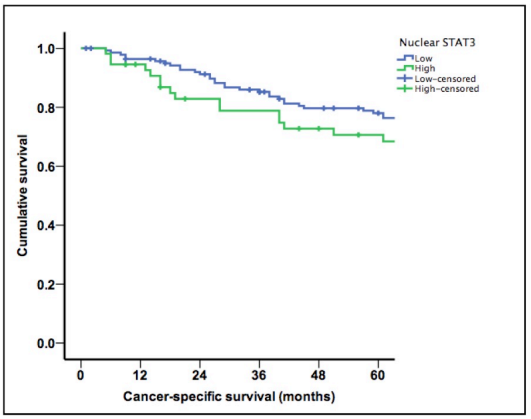


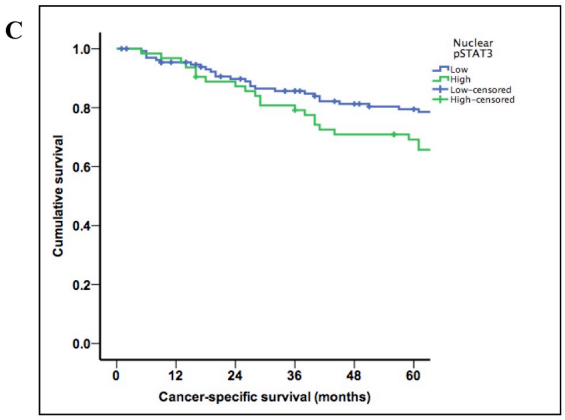
Figure 6.1 An example of STAT3 and phosphorylated STAT3 expression in patients with colorectal cancer (x200 magnification). **(A)** exhibits low tumour epithelial cell expression of STAT3, whereas **(B)** exhibits high tumour epithelial cell expression of STAT3. **(C)** exhibits low tumour epithelial cell expression of pSTAT3, whereas **(D)** exhibits high tumour epithelial cell expression of pSTAT3



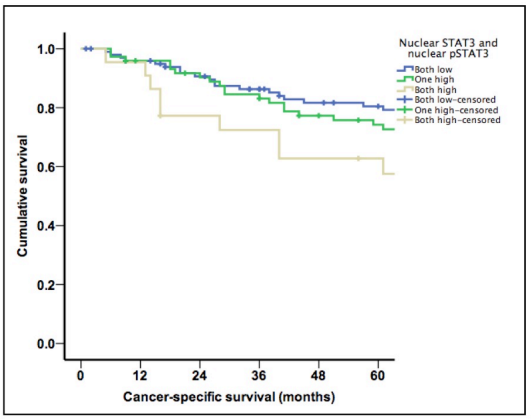
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low	132	125	115	105	93	89
High	64	57	50	47	42	38



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low	141	133	124	113	100	95
High	55	49	41	39	35	32



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low	133	121	110	102	92	87
High	63	61	55	50	43	40



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Both low	100	93	85	78	70	67
One high	74	68	64	59	52	48
Both high	22	21	16	15	13	12

Figure 6.2 The relationship between STAT3 and pSTAT3 expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer. **(A)** cytoplasmic STAT3 expression (*P*=0.068), **(B)** nuclear STAT3 expression (*P*=0.012), **(C)** nuclear pSTAT3 expression (*P*=0.116), and **(D)** combined nuclear STAT3/pSTAT3 expression (*P*=0.012). All *P*-values calculated using log-rank analysis

Table 6.1 The relationship between STAT3 and pSTAT3 expression and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

			Cytoplasmic STAT3				Nuclear STAT3				Nuclear pSTAT3			
Clinicopathological characteristics		**All *N*=196 (%)**	**Low *n*=76 (%)**	**Moderate *n*=56 (%)**	**High *n*=64 (%)**	***P***	**Low *n*=75 (%)**	**Moderate *n*=66 (%)**	**High *n*=55 (%)**	***P***	**Low *n*= 72 (%)**	**Moderate *n*=61 (%)**	**High *n*=63 (%)**	***P***
Host characteristics														
Age						0.571				0.199				0.026
	<65	72 (37)	23 (30)	22 (39)	27 (42)		29 (39)	25 (37)	18 (33)		19 (26)	26 (43)	27 (43)	
	65-74	61 (31)	29 (38)	18 (32)	14 (22)		25 (33)	22 (33)	14 (26)		23 (32)	19 (31)	19 (30)	
	>75	63 (32)	24 (32)	16 (29)	23 (36)		21 (28)	10 (29)	23 (41)		30 (42)	16 (26)	17 (27)	
Sex						0.833				0.647				0.906
	Female	94 (48)	40 (53)	21 (37)	33 (52)		35 (47)	31 (47)	28 (51)		38 (53)	23 (38)	33 (52)	
	Male	102 (52)	36 (47)	35 (63)	31 (48)		40 (53)	35 (53)	27 (49)		34 (47)	38 (62)	30 (48)	
Adjuvant						0.532				0.038				0.389
therapy	**No**	142 (72)	55 (72)	44 (79)	43 (67)		48 (64)	50 (76)	44 (80)		56 (78)	41 (67)	45 (71)	
	Yes	54 (28)	21 (28)	12 (21)	21 (33)		27 (36)	16 (24)	11 (20)		16 (22)	20 (33)	18 (29)	
Tumour characteristics														
Tumour						0.375				0.242				0.860
location	**Colon**	130 (66)	48 (63)	37 (66)	45 (70)		47 (63)	43 (65)	40 (73)		49 (68)	37 (61)	44 (70)	
	Rectum	66 (34)	28 (37)	19 (34)	19 (30)		28 (37)	23 (35)	15 (27)		23 (32)	24 (39)	19 (30)	
T stage						0.288				0.480				0.694
	1-2	25 (13)	10 (13)	9 (16)	6 (9)		10 (13)	10 (15)	5 (9)		10 (14)	8 (13)	7 (11)	
	3	121 (61)	49 (65)	34 (61)	38 (59)		46 (61)	41 (62)	34 (62)		43 (60)	39 (64)	39 (62)	
	4	50 (26)	17 (22)	13 (23)	20 (31)		19 (25)	15 (23)	16 (29)		19 (26)	14 (23)	17 (27)	
N stage						0.183				0.470				0.039
	0	110 (56)	47 (61)	30 (53)	33 (51)		34 (45)	46 (70)	30 (54)		47 (65)	33 (54)	30 (48)	
	1	68 (35)	24 (32)	20 (36)	24 (38)		34 (45)	16 (24)	18 (33)		21 (29)	21 (34)	26 (41)	
	2	18 (9)	5 (7)	6 (11)	7 (11)		7 (10)	4 (6)	7 (13)		4 (6)	7 (12)	7 (11)	
TNM stage						0.211				0.494				0.051
	I	16 (8)	6 (8)	7 (13)	3 (5)		6 (8)	8 (12)	2 (4)		7 (10)	5 (8)	4 (6)	
	II	94 (48)	41 (54)	23 (41)	30 (47)		28 (37)	38 (58)	28 (50)		40 (55)	28 (46)	26 (41)	
	III	86 (44)	29 (38)	26 (46)	31 (48)		41 (55)	20 (30)	25 (46)		25 (35)	28 (46)	33 (52)	

Table 6.1 (continued) The relationship between STAT3 and pSTAT3 expression and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

			Cytoplasmic STAT3				Nuclear STAT3				Nuclear pSTAT3			
Clinicopathological characteristics		**All *N*=196 (%)**	**Low *n*=76 (%)**	**Moderate *n*=56 (%)**	**High *n*=64 (%)**	***P***	**Low *n*=75 (%)**	**Moderate *n*=66 (%)**	**High *n*=55 (%)**	***P***	**Low *n*= 72 (%)**	**Moderate *n*=61 (%)**	**High *n*=63 (%)**	***P***
Tumour characteristics														
Tumour						0.530				0.108				0.174
differentiation	**Mod/well**	174 (89)	69 (91)	49 (87)	56 (87)		63 (84)	60 (91)	51 (93)		60 (83)	57 (93)	57 (91)	
	Poor	22 (11)	7 (9)	7 (13)	8 (13)		12 (16)	6 (9)	4 (7)		12 (17)	4 (7)	6 (10)	
Venous invasion						0.465				0.337				0.271
	No	129 (66)	51 (67)	39 (70)	39 (61)		46 (61)	45 (68)	38 (69)		51 (71)	39 (64)	39 (62)	
	Yes	67 (34)	25 (33)	17 (30)	25 (39)		29 (39)	21 (32)	17 (31)		21 (29)	22 (36)	24 (38)	
Margin						0.856				0.649				0.562
involvement	**No**	187 (95)	72 (95)	54 (96)	61 (95)		70 (93)	65 (98)	52 (94)		70 (97)	57 (93)	60 (95)	
	Yes	9 (5)	4 (5)	2 (4)	3 (5)		5 (7)	1 (2)	3 (6)		2 (3)	4 (7)	3 (5)	
Peritoneal						0.423				0.794				0.787
involvement	**No**	144 (3)	57 (75)	43 (77)	44 (69)		55 (73)	50 (76)	39 (71)		53 (74)	46 (75)	45 (71)	
	Yes	52 (27)	19 (25)	13 (23)	20 (31)		20 (27)	16 (24)	16 (29)		19 (26)	15 (25)	18 (29)	
Tumour						0.652				0.799				0.087
perforation	**No**	192 (98)	74 (97)	55 (98)	63 (98)		73 (97)	66 (100)	53 (96)		69 (96)	60 (98)	63 (100)	
	Yes	4 (2)	2 (3)	1 (2)	1 (2)		2 (3)	0 (0)	2 (4)		3 (4)	1 (2)	0 (0)	
Mismatch						0.741				0.406				0.491
repair status	**Competent**	169 (86)	65 (85)	48 (86)	56 (87)		62 (83)	59 (89)	48 (87)		61 (85)	52 (85)	56 (89)	
	Deficient	27 (14)	11 (15)	8 (14)	8 (13)		13 (17)	7 (11)	7 (13)		11 (15)	9 (15)	7 (11)	

Data analysed using χ^2 analysis for linear trend.

Table 6.2 The relationship between STAT3 and pSTAT3 expression and tumour microenvironment characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Cytoplasmic STAT3				Nuclear STAT3				Nuclear pSTAT3			
Tumour microenvironment (*n* when data missing)		**Low** ***n*=76 (%)**	**Moderate** ***n*=56 (%)**	**High** ***n*=64 (%)**	***P***	**Low** ***n*=75 (%)**	**Moderate** ***n*=66 (%)**	**High** ***n*=55 (%)**	***P***	**Low** ***n*= 72 (%)**	**Moderate** ***n*=61 (%)**	**High** ***n*=63 (%)**	***P***
Klintrup-Makinen					0.208				0.657				0.582
grade	**Weak**	28 (37)	20 (36)	17 (27)		25 (33)	24 (36)	16 (29)		26 (36)	19 (31)	20 (32)	
	Strong	48 (63)	36 (64)	47 (73)		50 (67)	42 (64)	30 (71)		46 (64)	42 (69)	43 (68)	
Tumour stroma					0.241				0.794				0.090
percentage	**Low**	59 (78)	43 (78)	44 (69)		56 (75)	51 (77)	39 (72)		55 (78)	50 (82)	40 (64)	
(195)	**High**	17 (22)	12 (22)	20 (31)		19 (25)	15 (23)	15 (28)		16 (22)	11 (18)	22 (36)	
CD3$^+$ margin density					0.332				0.055				0.648
(184)	**Low**	36 (49)	30 (60)	35 (57)		37 (51)	28 (46)	36 (71)		34 (54)	31 (52)	36 (58)	
	High	37 (51)	20 (40)	26 (43)		35 (49)	33 (54)	15 (29)		29 (46)	28 (48)	26 (42)	
CD3$^+$ cancer cell nest					0.012				0.262				0.150
density	**Low**	38 (51)	42 (79)	45 (70)		47 (64)	38 (59)	40 (74)		43 (62)	35 (58)	47 (75)	
(192)	**High**	37 (49)	11 (21)	19 (30)		27 (37)	26 (41)	14 (26)		26 (38)	25 (42)	16 (25)	
CD8$^+$ margin density					0.630				0.177				0.806
(184)	**Low**	41 (59)	34 (64)	33 (54)		38 (53)	37 (61)	33 (65)		38 (59)	33 (55)	37 (62)	
	High	29 (41)	19 (36)	28 (46)		34 (47)	25 (39)	18 (35)		26 (41)	27 (45)	23 (38)	
CD8$^+$ cancer cell nest					0.003				0.039				0.730
density	**Low**	41 (57)	45 (83)	51 (80)		47 (63)	47 (76)	43 (80)		50 (72)	41 (68)	46 (75)	
(190)	**High**	31 (43)	9 (17)	13 (20)		27 (37)	15 (24)	11 (20)		19 (28)	19 (32)	15 (25)	
CD45R0$^+$ margin					0.960				0.089				0.282
density	**Low**	38 (52)	27 (51)	31 (52)		33 (47)	31 (48)	32 (63)		32 (48)	29 (50)	37 (57)	
(186)	**High**	35 (48)	26 (49)	29 (48)		38 (54)	33 (52)	19 (37)		38 (52)	29 (50)	26 (43)	
CD45R0$^+$ cancer cell					0.408				0.268				0.037
density	**Low**	48 (64)	43 (80)	44 (70)		48 (67)	46 (70)	41 (76)		46 (64)	39 (67)	50 (81)	
(192)	**High**	27 (36)	11 (20)	19 (30)		24 (33)	20 (30)	13 (24)		26 (36)	19 (33)	12 (19)	
FOXP3$^+$ margin					0.180				0.373				0.466
density	**Low**	37 (51)	29 (56)	38 (62)		39 (53)	34 (54)	31 (62)		40 (60)	32 (54)	32 (53)	
(186)	**High**	36 (49)	23 (44)	23 (38)		34 (47)	29 (46)	19 (38)		27 (40)	27 (46)	28 (47)	
FOXP3$^+$ cancer cell					0.002				0.807				0.181
nest density	**Low**	26 (36)	26 (49)	39 (63)		39 (53)	25 (39)	27 (53)		38 (56)	26 (44)	27 (44)	
(188)	**High**	47 (64)	27 (51)	23 (37)		34 (47)	39 (61)	24 (47)		30 (44)	33 (56)	34 (56)	

Table 6.2 (continued) The relationship between tumour cell STAT3 and pSTAT3 expression and T-lymphocyte density of patients undergoing elective, potentially curative resection of stage I-III mismatch repair competent colorectal cancer
Data analysed using χ^2 analysis for linear trend.

		Cytoplasmic STAT3 h-score				Nuclear STAT3 h-score				Nuclear pSTAT3 h-score			
Tumour microenvironment (*n* when data missing)		**Low** ***n*=76 (%)**	**Moderate** ***n*=56 (%)**	**High** ***n*=64 (%)**	***P***	**Low** ***n*=75 (%)**	**Moderate** ***n*=66 (%)**	**High** ***n*=55 (%)**	***P***	**Low** ***n*= 72 (%)**	**Moderate** ***n*=61 (%)**	**High** ***n*=63 (%)**	***P***
CD3$^+$ margin					0.495				0.024				0.243
density	**Low**	32 (50)	26 (63)	29 (56)		31 (51)	23 (44)	33 (75)		27 (51)	27 (53)	33 (62)	
(157)	**High**	32 (50)	15 (37)	23 (44)		30 (49)	29 (56)	11 (25)		26 (49)	24 (47)	20 (38)	
CD3$^+$ cancer cell					0.061				0.498				0.193
nest density	**Low**	36 (55)	36 (86)	38 (70)		42 (69)	33 (61)	35 (76)		37 (66)	31 (61)	42 (78)	
(161)	**High**	29 (45)	6 (14)	16 (30)		19 (31)	21 (39)	11 (24)		19 (34)	20 (39)	12 (22)	
CD8$^+$ margin					0.759				0.355				0.299
density	**Low**	36 (59)	28 (67)	29 (56)		34 (57)	30 (59)	29 (66)		30 (57)	29 (57)	34 (67)	
(155)	**High**	25 (41)	14 (33)	23 (44)		26 (43)	21 (41)	15 (34)		23 (43)	22 (43)	17 (33)	
CD8$^+$ cancer cell					0.035				0.255				0.666
nest density	**Low**	38 (61)	38 (88)	42 (78)		42 (69)	40 (77)	36 (78)		42 (75)	35 (69)	41 (79)	
(159)	**High**	24 (39)	5 (12)	12 (22)		19 (31)	12 (23)	10 (22)		14 (25)	16 (31)	11 (21)	
CD45R0$^+$ margin					0.783				0.085				0.199
density	**Low**	35 (56)	23 (55)	27 (53)		28 (48)	28 (52)	29 (66)		28 (51)	24 (49)	33 (63)	
(156)	**High**	28 (44)	19 (45)	24 (47)		30 (52)	26 (48)	15 (34)		27 (49)	25 (51)	19 (37)	
CD45R0$^+$ cancer					0.882				0.449				0.077
cell density	**Low**	45 (69)	35 (81)	37 (70)		41 (69)	41 (73)	35 (76)		40 (68)	33 (67)	44 (83)	
(161)	**High**	20 (31)	8 (19)	16 (30)		18 (31)	15 (27)	11 (24)		19 (32)	16 (33)	9 (17)	
FOXP3$^+$ margin					0.463				0.819				0.720
density	**Low**	33 (52)	20 (49)	31 (60)		33 (55)	26 (49)	25 (58)		31 (56)	26 (52)	27 (53)	
(156)	**High**	30 (48)	21 (51)	21 (40)		27 (45)	27 (51)	18 (42)		24 (44)	24 (48)	24 (47)	
FOXP3$^+$ cancer cell					0.006				0.910				0.239
nest density	**Low**	23 (36)	19 (45)	32 (61)		32 (52)	19 (35)	23 (53)		31 (55)	20 (40)	23 (44)	
(158)	**High**	41 (64)	23 (55)	20 (39)		29 (48)	35 (65)	20 (47)		25 (45)	30 (60)	29 (56)	

Table 6.3 The relationship between STAT3 and pSTAT3 expression and systemic inflammatory responses of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Cytoplasmic STAT3				Nuclear STAT3				Nuclear pSTAT3			
Systemic inflammatory response (*n* when data missing)		**Low *n*=76 (%)**	**Moderate *n*=56 (%)**	**High *n*=64 (%)**	***P***	**Low *n*=75 (%)**	**Moderate *n*=66 (%)**	**High *n*=55 (%)**	***P***	**Low *n*= 72 (%)**	**Moderate *n*=61 (%)**	**High *n*=63 (%)**	***P***
Modified Glasgow Prognostic					0.004				0.244				0.651
Score	**0**	53 (70)	33 (59)	33 (51)		46 (61)	42 (64)	31 (56)		44 (61)	36 (59)	39 (62)	
	1	20 (26)	18 (32)	19 (30)		23 (31)	20 (30)	14 (26)		20 (28)	17 (28)	20 (32)	
	2	3 (4)	5 (9)	12 (19)		6 (8)	4 (6)	10 (18)		8 (11)	8 (13)	4 (6)	
Neutrophil count					0.515				0.676				0.470
(195)	**≤7.5x10^9/L**	67 (88)	47 (85)	54 (84)		63 (85)	60 (91)	45 (82)		60 (85)	52 (85)	56 (89)	
	>7.5x10^9/L	9 (12)	8 (15)	10 (16)		11 (15)	6 (9)	10 (18)		11 (16)	9 (15)	7 (11)	
Lymphocyte count					0.942				0.174				0.209
(195)	**>4x10^9/L**	76 (100)	54 (98)	64 (100)		74 (100)	66 (100)	54 (98)		71 (100)	61 (100)	62 (98)	
	≤4x10^9/L	0 (0)	1 (2)	0 (0)		0 (0)	0 (0)	1 (2)		0 (0)	0 (0)	1 (2)	
Platelet count					0.557				0.587				0.895
(176)	**≤400x10^9/L**	58 (87)	44 (86)	48 (83)		55 (85)	49 (83)	46 (88)		57 (85)	44 (85)	49 (86)	
	>400x10^9/L	9 (13)	7 (14)	10 (17)		10 (15)	10 (17)	6 (12)		10 (15)	8 (15)	8 (14)	
Neutrophil: lymphocyte ratio					0.350				0.131				0.352
(195)	**≤5**	62 (82)	45 (82)	48 (75)		61 (82)	55 (83)	39 (71)		56 (79)	45 (74)	54 (86)	
	>5	14 (18)	10 (18)	16 (25)		13 (18)	11 (17)	16 (29)		15 (21)	16 (26)	9 (14)	
Neutrophil: platelet score					0.441				0.831				0.602
(176)	**0**	52 (78)	40 (78)	40 (69)		47 (72)	46 (78)	39 (75)		49 (73)	39 (75)	44 (77)	
	1	13 (19)	7 (14)	17 (29)		16 (25)	10 (17)	11 (21)		15 (22)	11 (21)	11 (19)	
	2	2 (3)	5 (8)	1 (2)		2 (3)	3 (5)	2 (4)		3 (5)	2 (4)	2 (4)	

Data analysed using χ^2 analysis for linear trend.

Table 6.4 The relationship between STAT3 and pSTAT3 expression and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	N	5-year CSS % (SE)	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	P
Cytoplasmic STAT3						
Low expression	132	81 (3)			-	
High expression	64	67 (6)	1.62 (0.96-2.65)	0.072	-	
Nuclear STAT3						
Low expression	141	78 (4)			-	
High expression	55	70 (6)	1.89 (1.12-3.22)	0.018	-	
Nuclear pSTAT3						
Low expression	133	80 (4)			-	
High expression	63	69 (6)	1.52 (0.90-2.57)	0.119	-	
Combined cytoplasmic STAT3/ nuclear STAT3 (Model 1)						
Both low	106	81 (4)				
One high	61	73 (6)	1.56 (1.20-2.17)	0.009	-	0.221
Both high	29	63 (9)				
Combined cytoplasmic STAT3/ nuclear pSTAT3 (Model 2)						
Both low	95	80 (4)				
One high	75	79 (5)	1.50 (1.06-2.13)	0.024	-	0.526
Both high	26	54 (10)				
Combined nuclear STAT3/ nuclear pSTAT3 (Model 3)						
Both low	100	81 (4)				
One high	74	74 (5)	1.63 (1.14-2.34)	0.008	1.63 (1.14-2.34)	0.008
Both high	22	62 (11)				

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis. CSS – cancer-specific survival, SE – standard error, HR – hazard ratio, 95% CI – 95% confidence interval

Table 6.4 displays the relationship between markers of STAT3 expression and cancer-specific survival of patients undergoing elective resection of stage I-III colorectal cancer as measured by five-year survival and hazard ratios and 95% confidence intervals. The combination of nuclear STAT3 and pSTAT3 expression showed greatest prognostic value.

Table 6.5 The relationship between combined cytoplasmic and nuclear STAT3 expression and local and systemic environment characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Nuclear STAT3/ Nuclear pSTAT3			
Tumour microenvironment (*n* when data missing)		**Both low *n*=100 (%)**	**One high *n*=74 (%)**	**Both high *n*=22 (%)**	***P***
Klintrup-Makinen grade	**Weak**	35 (35)	24 (32)	6 (27)	0.486
	Strong	65 (65)	50 (68)	16 (73)	
Tumour stroma percentage (195)	**Low**	81 (81)	51 (69)	14 (67)	0.056
	High	19 (19)	23 (31)	7 (33)	
CD3$^+$ margin density (184)	**Low**	45 (48)	40 (58)	16 (73)	0.033
	High	48 (52)	29 (42)	6 (27)	
CD3$^+$ cancer cell nest density (192)	**Low**	58 (60)	47 (64)	20 (91)	0.017
	High	39 (40)	26 (26)	2 (9)	
CD8$^+$ margin density (184)	**Low**	53 (56)	40 (60)	15 (68)	0.295
	High	42 (44)	27 (40)	7 (32)	
CD8$^+$ cancer cell nest density (190)	**Low**	67 (69)	51 (72)	19 (86)	0.153
	High	30 (31)	20 (28)	3 (14)	
CD45R0$^+$ margin density (186)	**Low**	44 (46)	37 (54)	15 (68)	0.051
	High	52 (54)	31 (46)	7 (32)	
CD45R0$^+$ cancer cell density (192)	**Low**	66 (67)	47 (65)	22 (100)	0.030
	High	32 (33)	25 (35)	0 (0)	
FOXP3$^+$ margin density (186)	**Low**	55 (57)	35 (51)	14 (67)	0.747
	High	42 (43)	33 (49)	7 (33)	
FOXP3$^+$ cancer cell nest density (188)	**Low**	48 (50)	32 (46)	11 (52)	0.964
	High	49 (50)	38 (54)	10 (48)	
Modified Glasgow Prognostic Score	**0**	62 (62)	44 (59)	13 (59)	0.576
	1	29 (29)	22 (30)	6 (27)	
	2	9 (9)	8 (11)	3 (14)	
Neutrophil:lymphocyte ratio (195)	**≤5**	79 (80)	59 (80)	17 (77)	0.837
	>5	20 (20)	15 (20)	5 (23)	
Neutrophil:platelet score (176)	**0**	65 (73)	51 (76)	16 (76)	0.746
	1	18 (21)	16 (24)	3 (14)	
	2	5 (6)	0 (0)	2 (10)	

Data analysed using χ^2 analysis for linear trend.

Table 6.6 The relationship between combined cytoplasmic and nuclear STAT3 expression, clinicopathological characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.00 (0.73-1.37)	0.986	-	-
Sex (Female/ male)	1.43 (0.84-2.44)	0.188	-	-
Adjuvant therapy (No/ yes)	1.43 (0.83-2.47)	0.196	-	-
Tumour site (Colon/ rectum)	0.99 (0.57-1.74)	0.983	-	-
TNM stage (I /II /III)	2.16 (1.35-3.48)	0.001	-	0.228
Tumour differentiation (Mod-well/ poor)	1.18 (0.51-2.75)	0.700	-	-
Venous invasion (No/ yes)	3.35 (1.97-5.70)	<0.001	2.82 (1.58-5.04)	<0.001
Margin involvement (No/ yes)	2.82 (1.12-7.09)	0.028	-	0.282
Peritoneal involvement (No/ yes)	2.45 (1.45-4.13)	0.001	-	0.103
Tumour perforation (No/ yes)	4.34 (1.04-18.11)	0.044	-	0.106
Modified Glasgow Prognostic Score (0/ 1/ 2)	1.43 (0.99-2.08)	0.057	1.79 (1.18-2.70)	0.006
NPS (0/ 1/ 2)	1.72 (1.13-2.62)	0.012	-	0.098
NLR (<5/ >5)	1.13 (0.60-2.13)	0.715	-	-
Mismatch repair status (Competent/ deficient)	1.37 (0.69-2.71)	0.370	-	-
Klintrup-Makinen grade (High/ low)	2.33 (1.20-4.49)	0.012	2.23 (1.04-4.81)	0.040
Tumour stroma percentage (Low/ high)	2.52 (1.48-4.30)	0.001	2.75 (1.55-4.89)	0.001
Nuclear STAT3/ nuclear pSTAT3 (Both low/ one high/ both high)	1.63 (1.14-2.34)	0.008	1.39 (0.94-2.06)	0.102

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

7 Staging the tumour and staging the host: evaluation of a novel tumour microenvironment-based prognostic score in patients with primary operable colorectal cancer

7.1 Introduction

In Chapter 4, H&E-based assessment of the tumour-associated stroma, using TSP, was shown to have prognostic value independent of the clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. Furthermore, assessment of TSP held prognostic value independent of other components of the tumour microenvironment, and in particular the generalised inflammatory cell infiltrate as measured using the KM grade.

Despite appearing to hold independent prognostic value, combined assessment of the tumour-associated stroma (using TSP) and tumour inflammatory cell infiltrate (using KM grade), and subsequently the interaction and combined impact on survival of patients with primary operable colorectal cancer, has not previously been examined. This presents the opportunity to develop a tumour microenvironment-based score which may provide prognostic information complimentary to current clinicopathological assessment. Therefore, the aim of the present study was to examine the combined prognostic value of KM grade and TSP, and to evaluate a novel, tumour microenvironment-based prognostic score in patients with stage I-III colorectal cancer undergoing elective, potentially curative resection.

7.2 Patients and Methods

Clinicopathological characteristics

Patients who had undergone elective, potentially curative resection of stage I-III colorectal adenocarcinoma without neoadjuvant therapy were identified from a prospectively collected and maintained database of elective and emergency colorectal cancer resections performed in a single surgical unit at GRI . Inclusion and exclusion criteria and clinicopathological staging has previously been described in Chapter 4. Determination of MMR status, as previously described in Chapter 5, was performed for a subset of patients who were concurrently included in a previously constructed TMA.

Multi-disciplinary team review, indications for adjuvant chemotherapy and routine follow-up of patients following surgery has previously been described in Chapter 2. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 15th March 2013 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Assessment of the tumour microenvironment

Using archived H&E-stained sections of the deepest point of invasion, the generalised inflammatory cell infiltrate was assessed using KM grade and the tumour-associated stroma was assessed using TSP as previously described in Chapter 4. Briefly, KM grade was classified as low grade or high grade and TSP was classified as low (≤50%) or high (>50%).

Statistical analysis

The relationship between clinicopathological and tumour microenvironment characteristics and survival was examined using univariate Cox proportional hazards regression to

calculate HRs and 95% CIs. Variables with *P*-value <0.1 on univariate regression analysis were examined in a multivariable model using a backwards conditional method. The relationship between a tumour microenvironment-based score and survival was further examined using Kaplan-Meier log-rank analysis, with five-year survival presented as percentage surviving (SE). The relationship between the tumour microenvironment score and other clinicopathological characteristics was examined using the $\chi 2$ test for linear trend. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). The West of Scotland Research Ethics Committee approved the study.

7.3 Results

A total of 307 patients who underwent elective resection for stage I-III colorectal cancer were included. Clinicopathological characteristics are summarised in Table 7.1. Two thirds of patients were older than 65 at time of surgery with a similar number of males and females. The majority of patients (71%) underwent colonic resection, with pathological confirmation of lymph node negative (stage I/II) disease in approximately two thirds of patients. Overall, 82 patients (27%) received adjuvant chemotherapy; 59 patients (52%) with lymph node positive (stage III) disease received adjuvant chemotherapy compared to 23 patients (12%) with lymph node negative (stage I/II) colorectal cancer. Mismatch repair status was available for 208 patients, with MMR deficient colorectal cancer identified in 33 patients (16%). A low KM grade and high TSP were identified in 66% and 25% of patients respectively.

The median follow-up of survivors was 126 months (range 59-194 months), with 95 cancer-specific deaths and 86 non-cancer deaths. Five-year cancer specific survival was 75% overall, 85% in patients with stage I/II disease and 58% in patients with stage III disease. The relationship between clinicopathological and tumour microenvironment characteristics and cancer-specific survival is shown in Table 7.1. On univariate analysis, advancing age ($P<0.05$), T stage ($P<0.01$), N stage, venous invasion (both $P<0.001$), margin involvement ($P<0.05$), peritoneal involvement ($P=0.001$), low KM grade and high TSP (both $P=0.001$) were all associated with reduced survival. Mismatch repair deficiency showed a trend towards increased survival ($P=0.082$). On multivariate survival analysis, the presence of venous invasion ($P=0.001$), a low KM grade ($P<0.05$) and a high TSP ($P<0.01$) were independently associated with poorer cancer-specific survival, whereas advanced age and increasing N stage showed a trend towards poorer survival ($P=0.052$ and $P=0.061$ respectively).

The prognostic value of KM grade and TSP was further examined (Table 7.2). Five-year cancer-specific survival of patients was stratified from 90% to 68% by KM grade, and from 80% to 62% by TSP. A cumulative prognostic score based on these characteristics of the tumour microenvironment was subsequently derived. As the univariate HRs and 95% CIs for low KM grade and high TSP overlapped, the presence of each characteristic scored one point, thus stratifying patients into four possible groups. Patients with a high KM grade and low TSP comprised 27% of the study population and had a five-year survival of 89%; conversely patients with a low KM grade and high TSP comprised 19% of the group, with a four-fold increased risk of cancer-death and five-year survival of 51%. The presence of a low KM grade and low TSP was identified in almost half of the patients studied and was associated with an intermediate five-year survival of 75% and two-fold increased risk of cancer-death compared to those patients with high KM grade and low TSP. Only 6% of patients had a high KM grade with a high TSP; this group of patients had an identical five-year survival to patients with a high KM grade and low TSP.

As a high TSP was not associated with poorer cancer-specific survival in patients with a high KM grade, the cumulative prognostic score was modified to include all patients with a high KM grade in the good prognostic group, irrespective of TSP assessment. This modified prognostic score, termed the Glasgow Microenvironment Score (GMS), stratified patients with primary operable colorectal cancer into three distinct prognostic groups (Figure 7.1, Table 7.2): a good prognostic group (GMS=0 with a high KM grade and either high or low TSP) with five-year survival of 89%, an intermediate prognostic group (GMS=1 with a low KM grade and low TSP) with an almost two-fold increased risk of cancer death and five-year survival of 75%, and a poor prognostic group (GMS=3 with a low KM grade and high TSP) with a four-fold increased risk of death and five-year survival of 51%. Furthermore, on multivariate analysis (Table 7.3), GMS was associated with a two-fold increased risk of cancer-death (HR 1.93, 95% CI 1.36-2.74, $P<0.001$),

independent of N stage (P<0.05) and venous invasion (P=0.001). Examples of the microenvironment typical of GMS 0 and GMS 2 are displayed in Figure 7.2.

The clinical utility of the GMS was further explored in relation to lymph node involvement, venous invasion, MMR status and use of adjuvant therapy (Table 7.4). The GMS stratified survival of patients with both lymph node negative (stage I/II) and positive (stage III) disease (P=0.036 and P=0.002, respectively). Using the combination of lymph node involvement and GMS, five-year cancer-specific survival ranged from 92% (stage I/II and GMS=0) to 37% (stage III and GMS=2). Furthermore, patients with stage III disease and GMS=0 had five-year survival superior to that of patients with stage I/II disease and GMS=2 (81% versus 69%). The GMS was similarly able to provide further prognostic information alongside venous invasion and MMR status; the combination of venous invasion and GMS stratified five-year survival from 93% (venous invasion absent and GMS=0) to 27% (venous invasion present and GMS=2), whereas using the combination of MMR status and GMS, five-year survival ranged from 100% (MMR deficient and GMS=0) to 37% (MMR competent and GMS=2). In addition, when patients were stratified by use of adjuvant therapy, GMS was predictive of survival independent of adjuvant therapy use (both P=0.002)

The relationship between GMS and clinicopathological characteristics was subsequently examined (Table 7.5). Increasing GMS was associated with use of adjuvant chemotherapy (P<0.05), increasing TNM stage, T stage (both $P\leq$0.001), N stage (P<0.01), venous invasion (P<0.05), margin and peritoneal involvement (P<0.01). The GMS was not associated with age, sex, tumour site, differentiation, MMR status or the presence of tumour perforation.

7.4 Discussion

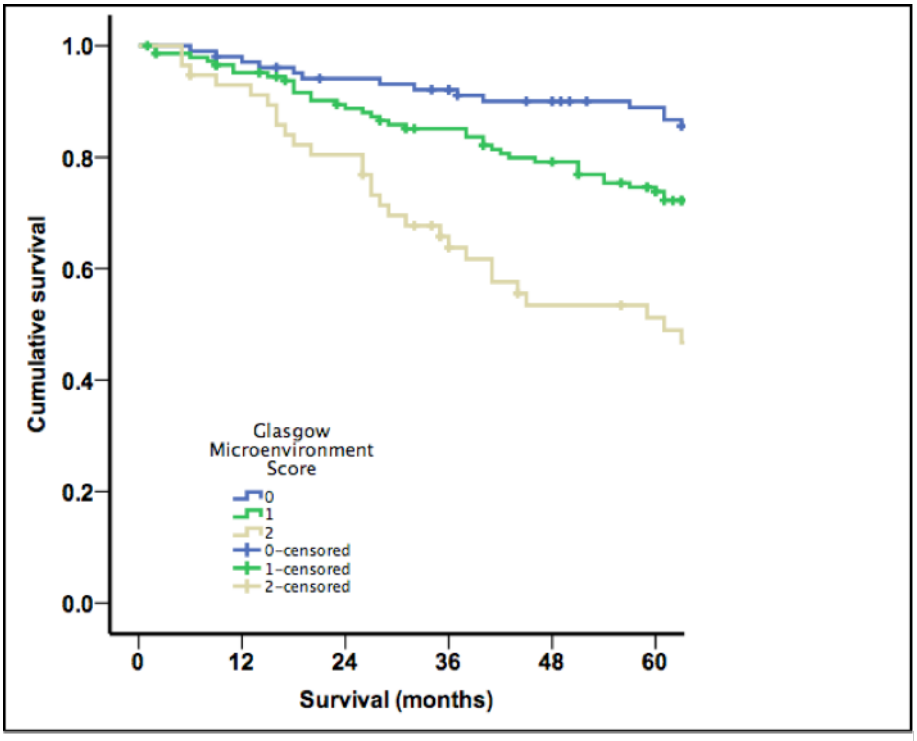
The present study, for the first time, examines the clinical utility of combined assessment of the tumour inflammatory cell infiltrate and tumour stroma, utilising the KM grade and TSP respectively, in patients with primary operable colorectal cancer. A simple, cumulative prognostic score based on the assessment and interaction of these characteristics using routine histopathological specimens and termed the Glasgow Microenvironment Score (GMS), was able to provide improved risk stratification. Utilising this score, it was possible to identify a group of patients with lymph node negative disease with five-year survival comparable to patients with lymph node involvement. Conversely, it was also possible to identify patients with stage III disease and five-year survival of over 80%. Similarly, the GMS was able to stratify patient survival independent of venous invasion and MMR status. Such a simple, routinely available score can be readily evaluated and validated. If this proves to be the case, then the GMS may help better inform decisions regarding the need for adjuvant therapy and surveillance for otherwise "low risk" patients, or avoid unnecessary treatment for those previously deemed "high risk" on the basis of standard pathological staging.

The results of the present study also have implications regarding our understanding of the nature of the tumour microenvironment. As survival of patients with a strong KM grade did not differ with TSP, it could be inferred that the presence of a strong, conspicuous inflammatory infiltrate represents the host's normal anti-tumour response. Furthermore, few patients had a high TSP in the presence of a strong inflammatory cell infiltrate. As such, it may be loss of this coordinated immune response that facilitates disease progression, allowing tumour stroma formation that in turn facilitates growth and invasion. Therefore, future work must not only consider the intrinsic properties of the tumour cell itself, but also the components of the tumour microenvironment.

The results of the present study are limited by the small number of patients with stage I disease (21 patients), and as such it was not possible to examine the clinical utility of the GMS in this subgroup of patients separately. Given that earlier, node negative disease is likely to predominate with the introduction of screening (496), this would be an important area for further research. In addition, although the GMS stratified survival independent of MMR status, no other prognostic molecular markers were examined. To date however, few of these markers have been recommended for use in routine clinical practice, and as such their clinical utility in the management of patients with primary operable colorectal cancer is yet to be realised (497). Finally, the results of the present study remain to be validated. Separation of the study cohort into a training and validation set would have allowed for internal validation, however such an approach may have lacked sufficient statistical power for exploratory subgroup analysis. Furthermore, external validation would still be required before the GMS could be incorporated into routine pathological reporting. Given that the GMS utilises routine H&E-stained pathological specimens, this will facilitate external validation. Indeed, assessment of the GMS may be readily automated (464, 498), further facilitating validation and the implementation of such measures into routine clinical practice.

In summary, the present study demonstrates the clinical utility of a novel cumulative prognostic score based on the tumour inflammatory cell infiltrate and tumour stroma in patients with primary operable colorectal cancer. This score, termed the Glasgow Microenvironment Score, has much to commend it since it is simple and routinely available.

.



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
GMS 0	103	100	94	91	85	80
GMS 1	147	136	124	115	106	95
GMS 2	57	52	45	33	25	23

Figure 7.1 The relationship between the Glasgow Microenvironment Score and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer (log-rank $P<0.001$)

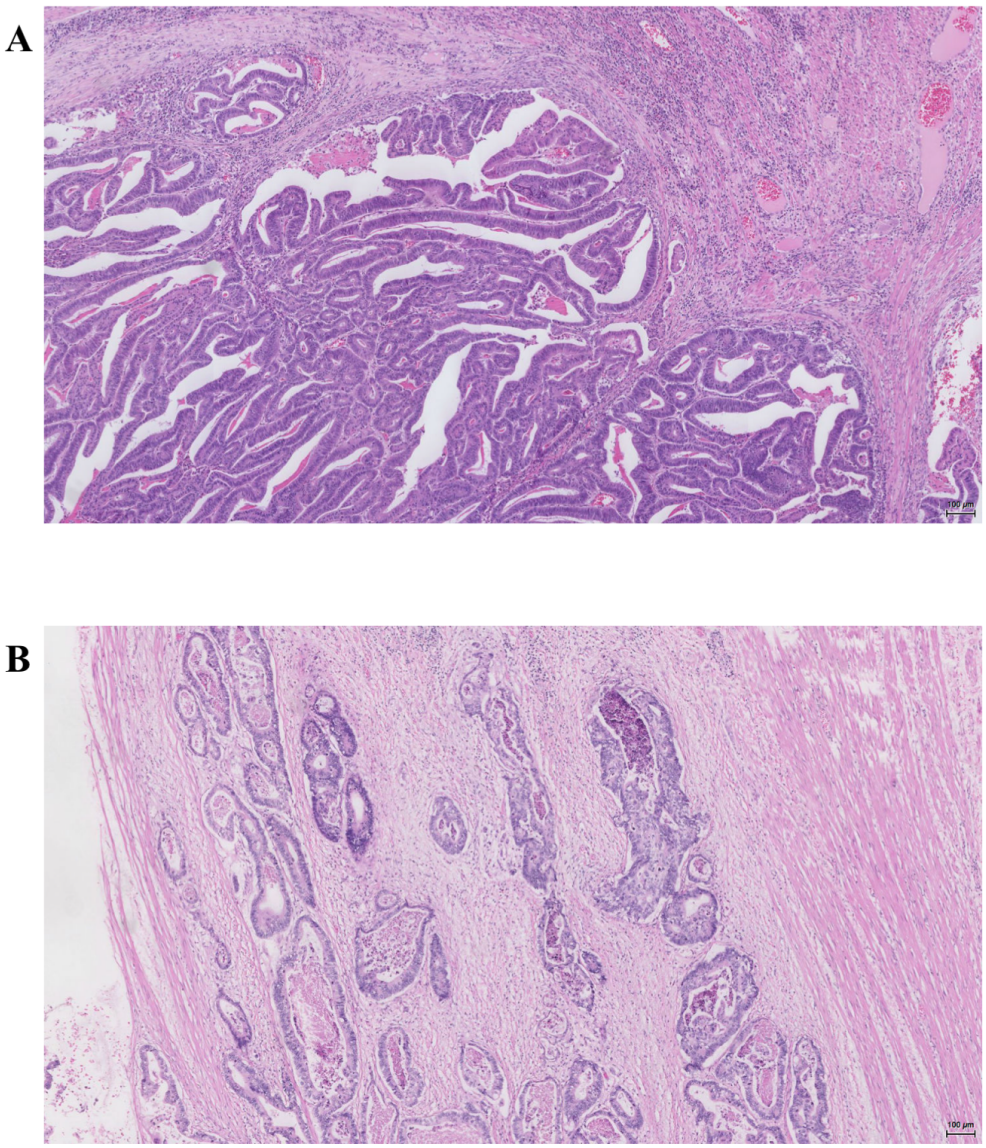


Figure 7.2 The Glasgow Microenvironment Score in patients with colorectal cancer (x200 magnification). **(A)** displays a high Klintrup-Mäkinen grade and low tumour stroma percentage(Glasgow Microenvironment Score 0), and **(B)**, displays a low Klintrup-Mäkinen grade and high tumour stroma percentage (Glasgow Microenvironment Score 2)

Table 7.1 The relationship between clinicopathological characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		Cancer-specific survival			
Clinicopathological characteristics	***n* (%)**	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	106 (35)/ 106 (35) / 95 (31)	1.36 (1.05-1.75)	0.018	-	0.052
Sex (Female/ male)	151 (49) / 156 (51)	0.92 (0.61-1.37)	0.667	-	-
Adjuvant therapy (No/ yes)	225 (73) / 82 (27)	1.26 (0.82-1.95)	0.289	-	-
Tumour site (Colon/ rectum)	218 (71) / 89 (29)	1.02 (0.65-1.59)	0.947	-	-
TNM stage (I /II /III)	21 (7) / 173 (56) / 113 (37)	-	-	-	-
T stage (1-2/ 3/ 4)	29 (9) / 196 (64) / 82 (27)	1.51 (1.12-2.05)	0.007	-	0.680
N stage (0/ 1/ 2)	194 (63) / 90 (29) / 23 (7)	1.96 (1.48-2.58)	<0.001	-	0.061
Tumour differentiation (Mod-well/ poor)	270 (88) / 37 (12)	1.60 (0.91-2.83)	0.104	-	-
Venous invasion (No/ yes)	203 (66) / 104 (34)	2.31 (1.54-3.47)	<0.001	2.41 (1.43-4.07)	0.001
Margin involvement (No/ yes)	289 (94) / 18 (6)	2.42 (1.22-4.82)	0.012	-	0.432
Peritoneal involvement (No/ yes)	229 (75) / 78 (25)	2.02 (1.33-3.06)	0.001	-	0.249
Tumour perforation (No/ yes)	300 (98) / 7 (2)	2.47 (0.777-7.84)	0.126	-	-
Mismatch repair status (Competent/ deficient) (208)	175 (84) / 33 (16)	0.47 (0.21-1.10)	0.082	-	0.352
Klintrup-Mäkinen grade (High grade/ low grade)	103 (34) / 204 (66)	2.42 (1.47-4.01)	0.001	2.02 (1.11-3.70)	0.021
Tumour stroma percentage (≤50%/ >50%)	231 (75) / 76 (25)	2.05 (1.35-3.12)	0.001	2.13 (1.27-3.58)	0.004

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 7.2 The relationship between Klintrup-Mäkinen grade, tumour stroma percentage and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

Clinicopathological characteristics	*N*	5-year CSS % (SE)	Univariate HR (95% CI)	*P*
Klintrup-Mäkinen grade				
KM high grade	103	90 (3)	1	
KM low grade	204	68 (3)	2.42 (1.47-4.01)	0.001
Tumour stroma percentage				
TSP low	231	80 (3)	1	
TSP high	76	62 (6)	2.05 (1.35-3.12)	0.001
Combined Klintrup-Mäkinen grade/ tumour stroma percentage				
KM high grade/ TSP low	84	89 (4)	1	-
KM high grade/ TSP high	19	89 (7)	1.23 (0.41-3.71)	0.715
KM low grade/ TSP low	147	75 (4)	2.00 (1.12-3.58)	0.020
KM low grade/ TSP high	57	51 (7)	4.25 (2.28-7.92)	<0.001
Glasgow Microenvironment Score				
0 (KM high grade)	103	89 (3)	1	-
1 (KM low grade/ TSP low)	147	75 (4)	1.92 (1.13-3.28)	0.017
2 (KM low grade/ TSP high)	57	51 (7)	4.08 (2.29-7.27)	<0.001

Data analysed using Cox proportional hazards regression to calculate univariate hazard ratios and 95% confidence intervals. CSS – cancer-specific survival, SE – standard error, HR – hazard ratio, 95% CI – 95% confidence interval,

Table 7.2 displays the relationship between KM grade and TSP, and scores based upon these characteristics, on cancer-specific survival of patients undergoing elective resection of stage I-III colorectal cancer as measured by five-year survival and hazard ratios and 95% confidence intervals. The combination of KM grade and TSP provided greater prognostic value than either characteristic alone.

Table 7.3 The relationship between the Glasgow Microenvironment Score, clinicopathological characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.36 (1.05-1.75)	0.018	-	0.068
Sex (Female/ male)	0.92 (0.61-1.37)	0.667	-	-
Adjuvant therapy (No/ yes)	1.26 (0.82-1.95)	0.289	-	-
Tumour site (Colon/ rectum)	1.02 (0.65-1.59)	0.947	-	-
TNM stage (I /II /III)	-	-	-	-
T stage (1-2/ 3/ 4)	1.51 (1.12-2.05)	0.007	-	0.685
N stage (0/ 1/ 2)	1.96 (1.48-2.58)	<0.001	1.45 (1.02-2.06)	0.040
Tumour differentiation (Mod-well/ poor)	1.60 (0.91-2.83)	0.104	-	-
Venous invasion (No/ yes)	2.31 (1.54-3.47)	<0.001	2.39 (1.42-4.01)	0.001
Margin involvement (No/ yes)	2.42 (1.22-4.82)	0.012	-	0.429
Peritoneal involvement (No/ yes)	2.02 (1.33-3.06)	0.001	-	0.230
Tumour perforation (No/ yes)	2.47 (0.777-7.84)	0.126	-	-
Mismatch repair status (Competent/ deficient) (208)	0.47 (0.21-1.10)	0.082	-	0.296
Glasgow Microenvironment Score (0/ 1/ 2)	2.03 (1.52-2.71)	<0.001	1.93 (1.36-2.74)	<0.001

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

.

Table 7.4 The relationship between the Glasgow Microenvironment Score, lymph node status, venous invasion, adjuvant chemotherapy and mismatch repair status and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

			Glasgow Microenvironment Score						
	All patients		0		1		2		*P*
	N	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	
Lymph node status									
Negative (Stage I/II)	173	86 (3)	70	92 (3)	99	84 (4)	25	69 (10)	0.036
Positive (Stage III)	113	58 (5)	33	81 (7)	48	55 (7)	32	37 (9)	0.002
Venous invasion									
Absent	203	82 (3)	74	93 (3)	98	77 (4)	31	70 (8)	0.025
Present	104	62 (5)	29	78 (8)	49	70 (7)	26	27 (9)	<0.001
Adjuvant treatment									
No adjuvant therapy	225	76 (3)	79	90 (3)	112	73 (4)	34	58 (9)	0.002
Adjuvant therapy	82	72 (5)	24	87 (7)	35	81 (7)	23	43 (10)	0.002
All patients (*n*=307)	307	75 (3)	103	89 (3)	147	75 (4)	57	51 (7)	<0.001
MMR status									
MMR deficient	33	84 (7)	13	100 (0)	15	67 (12)	5	-	<0.001
MMR competent	175	71 (4)	59	84 (5)	81	76 (5)	35	37 (9)	0.094
All patients (*n*=208)	208	73 (3)	72	87 (4)	96	75 (5)	40	45 (8)	<0.001

Log-rank *P*-value provided for the differential prognostic value of GMS in stratifying cancer-specific survival within each subgroup (rows). CSS – cancer-specific survival, SE – standard error.

Table 7.5 The relationship between Glasgow Microenvironment Score and clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer

		GMS 0	GMS 1	GMS 2	
Clinicopathological Characteristics		***n*=103 (%)**	***n*=147 (%)**	***n*=57 (%)**	***P***
Host characteristics					
Age					
	<65	36 (35)[a]	47 (32)	23 (40)	0.972
	65-74	39 (38)	50 (34)	17 (30)	
	>75	28 (27)	50 (34)	17 (30)	
Sex					0.386
	Female	51 (50)	77 (52)	23 (40)	
	Male	52 (51)	70 (48)	34 (60)	
Adjuvant therapy					0.040
	No	79 (77)	112 (76)	34 (60)	
	Yes	24 (23)	35 (24)	23 (40)	
Tumour characteristics					
Tumour site					0.812
	Colon	74 (72)	104 (71)	40 (70)	
	Rectum	29 (28)	43 (29)	17 (30)	
T stage					<0.001
	1-2	19 (18)	9 (6)	1 (2)	
	3	63 (61)	105 (71)	28 (49)	
	4	21 (20)	33 (22)	28 (49)	
N stage					0.004
	0	70 (68)	99 (67)	25 (44)	
	1	29 (28)	36 (25)	25 (44)	
	2	4 (4)	12 (8)	7 (12)	
TNM stage					0.001
	I	12 (12)	8 (5)	1 (2)	
	II	58 (56)	91 (62)	24 (42)	
	III	33 (32)	48 (33)	32 (56)	
Tumour differentiation					0.893
	Mod/well	90 (87)	131 (89)	49 (86)	
	Poor	13 (13)	16 (11)	8 (14)	
Venous invasion					0.032
	No	74 (72)	98 (67)	31 (54)	
	Yes	29 (28)	49 (33)	26 (46)	
Margin involvement					0.003
	No	101 (98)	139 (95)	49 (86)	
	Yes	2 (2)	8 (5)	8 (14)	
Peritoneal involvement					0.004
	No	83 (81)	113 (77)	33 (58)	
	Yes	20 (19)	34 (23)	24 (42)	
Tumour perforation					0.979
	No	101 (98)	143 (97)	56 (98)	
	Yes	2 (2)	4 (3)	1 (2)	
Mismatch repair status (*n*=208)					0.441
	Competent	59 (82)	81 (84)	35 (88)	
	Deficient	13 (18)	15 (16)	5 (13)	

Data analysed using χ^2 analysis for linear trend.

8 Tumour invasiveness as a determinant of the local and systemic environment and the basis of staging systems based on these characteristics in primary operable colorectal cancer

8.1 Introduction

For patients without overt systemic metastatic disease, prognosis and the need for adjuvant chemotherapy is primarily determined by the depth of invasion of the primary tumour (T stage) as well as the presence of regional lymph node metastases (N stage). However, the use of the TNM staging system remains problematical, since increasing disease stage does not necessarily reflect a stepwise increase in the risk of recurrence or death. For example, the survival of patients with Stage IIIa (T1/2, N1) colon cancer is superior to that of patients with stage IIb (T4, N0) disease (162). Given the failings of TNM criteria, there has been increasing effort to refine colorectal cancer staging using both pathological and molecular characterisation, particularly in the context of stage II and stage III disease (204, 432, 499). The local and systemic environment, as examined in previous Chapters, similarly reflects a promising approach.

The presence of adverse local and systemic characteristics is associated with increasing tumour invasiveness as determined by T stage. For example, in Chapter 2, the presence of an elevated systemic inflammatory response as measured by mGPS was shown to be associated with advancing T stage; only 2% of patients with T1 disease had a mGPS=2 compared to 41% of patients with T4 disease ($P<0.001$). Similarly, in Chapter 4, increasing T stage was associated with an increase in the proportion of patients with a high TSP ($P=0.027$). Furthermore, previous studies have shown that the density of both the generalised and adaptive T-lymphocytic inflammatory cell infiltrate degrades with increasing T stage (243, 433, 500).

Given the routine reporting of tumour invasiveness as measured by T stage, it would be of interest to examine the prognostic value of such measures of the local and systemic environment in comparison to present TNM-based staging. As such, the aim of the present study was to examine the interrelationships between T stage, components of the local and systemic environment, and survival of patients undergoing potentially curative resection of primary operable colorectal cancer.

8.2 Patients and Methods

Clinicopathological characteristics

Patients who had undergone elective, potentially curative resection of stage I-III colorectal adenocarcinoma without neoadjuvant therapy were identified from a prospectively collected and maintained database of all elective and emergency colorectal cancer resections performed in a single surgical unit at GRI. Inclusion and exclusion criteria, clinicopathological staging and the multi-disciplinary team process has previously been described in Chapter 4. Mismatch repair status was determined as described in Chapter 5.

Routine follow-up of patients following surgery has previously been described in Chapter 2. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Assessment of the tumour microenvironment

Using archived H&E sections of the deepest point of invasion, the generalised inflammatory cell infiltrate was assessed using KM grade and the tumour-associated stroma was assessed using TSP as previously described in Chapter 4. The KM grade was classified as low grade or high grade and TSP was classified as low (≤50%) or high (>50%). The GMS was calculated as described in Chapter 7. Tumour necrosis was graded as low (absent or <10% of tumour area) or high (>10% of tumour area) as previously decribed (235).

The adaptive T-lymphocytic infiltrate, as measured by mature ($CD3^+$) and cytotoxic ($CD8^+$) T-lymphocyte density within the invasive margin and cancer cell nests was examined as described in Chapter 4. Briefly, the density of $CD3^+$ and CD8+ T-

lymphocytes within each compartment was graded as either high (moderate or high) or low (weak or absent). The Immunoscore, a prognostic score based on the density of mature and cytotoxic T-lymphocytes within the invasive margin and cancer cell nests (501), was subsequently calculated. The Immunoscore ranged from Im0 (low density of both cell types in both regions) to Im4 (high density of both cell types in both regions). For the purposes of statistical analysis, patients were stratified into three prognostic groups: Im0/1 (low density), Im2/3 (moderate density) and Im4 (high density).

Assessment of systemic inflammatory responses

The mGPS was calculated as previously described in Chapter 2, and the NLR was calculated as described in Chapter 5.

Statistical analysis

The relationship between T stage and characteristics of the local and systemic environment was examined using the χ^2 test for linear trend. Their relationship with cancer-specific survival was examined using Kaplan-Meier log-rank analysis and was measured as percentage surviving at five years (SE) A *P*-value≤0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA).

8.3 Results

A total of 331 patients were included in the final analysis. The clinicopathological characteristics of this cohort have previously been described in Table 4.1. Two thirds of patients were 65 or older at time of surgery and 52% were male. Thirty percent of patients underwent resection of rectal cancer. Eighty-two patients (25%) received adjuvant therapy; 1 patient with stage I disease, 22 with stage II and 59 with stage III received adjuvant therapy. The majority of patients had a tumour breaching through muscularis propria, with 208 patients with T3 and 90 patients with a T4 tumour. Of the remaining patients, eight had a T1 tumour and 25 had a T2 tumour. Examples of T1, T3 and T4 tumours are displayed in Figure 8.1.

The relationship between T stage and clinicopathological characteristics is displayed in Table 8.1. Advancing T stage was associated with a colonic primary ($P<0.001$), advancing N stage ($P<0.01$), margin involvement and venous invasion (both $P<0.001$), and poor differentiation ($P<0.05$). In addition, advancing T stage was associated with adjuvant chemotherapy ($P<0.05$) but not age or sex. Mismatch repair status was available for 209 patients; MMR status was not associated with advancing T stage.

The relationship between T stage and components of the local and systemic environment was subsequently examined (Table 8.2). Advancing T stage was associated with high grade necrosis, an infiltrative invasive margin, low KM grade (all $P\leq0.001$) and high TSP ($P<0.01$). Furthermore, increasing T stage was associated with lower Immunoscore ($P<0.05$) and the presence of elevated systemic inflammatory responses as measured by both mGPS and NLR (both $P<0.05$). Certain characteristics appeared to become more prevalent earlier than others; for example, there was a proportionally greater increase in the number of patients with high grade necrosis, an infiltrative margin and low KM grade observed in the increase from T2 to T3, whereas the proportion of patients with high TSP

and elevated systemic inflammatory responses, showed a greater increase between T3 to T4 (Figure 8.2).

The interrelationships between components of the local and systemic environment was subsequently examined (Table 8.4); tumour necrosis was inversely associated with TSP and the Immunoscore and positively associated with the mGPS and NLR; an infiltrative margin was associated with necrosis and TSP; KM grade was positively associated with Immunoscore and inversely associated with TSP and NLR. Finally, mGPS and NLR were significantly associated.

The relationship between characteristics of the local and systemic environment and five-year cancer-specific survival was examined (Table 8.4). The median follow-up of survivors was 134 months (interquartile range 108-170 months) with 96 cancer deaths and 105 non-cancer deaths. Five-year cancer-specific survival of the whole cohort was 77%. N stage, character of the margin, KM grade, TSP, Immunoscore and mGPS all stratified five-year survival (all $P<0.001$). The GMS effectively stratified survival at five years from 90% to 53% ($P<0.001$). Tumour necrosis and the NLR did not stratify survival. Furthermore, MMR status was not statistically associated with survival.

To examine how such assessment may be utilised alongside T stage, subsequent survival analysis was performed in patients with T3 tumours. N stage, character of the margin, KM grade, TSP, GMS, Immunoscore and the mGPS all stratified five-year survival. Furthermore, NLR showed a trend towards an association with survival. In patients with T3 tumours, the GMS, the Immunoscore and mGPS had similar if not greater prognostic utility than N stage (Figure 8.3); the absolute difference in survival at five-years observed with N stage was 24%, whereas the absolute difference with GMS, Immunoscore and mGPS was 35%, 30% and 24% respectively.

8.4 Discussion

The results of the present study confirm the relationship between tumour invasion and the presence of adverse characteristics within both the local and systemic environment. Furthermore, how assessment of such characteristics provides an alternative staging system to the current TNM-based assessment has been examined.

Advancing T stage was shown to correlate significantly with the presence of an increasingly tumour-supportive microenvironment as evidenced by loss of host immune responses, expansion of the tumour-associated stroma and the presence of an infiltrative margin. It was of interest that the progression of each of these characteristics appeared to occur in a stepwise manner, with the proportion of some appearing to increase at an earlier T stage than others. For example, attenuation of the generalised local inflammatory cell infiltrate appeared to occur at a relatively early stage, with a strong KM grade present in 67% of patients with T2 tumours compared to only 32% of patients with T3 tumours. Conversely, the presence of an infiltrative margin and expansion of the tumour-associated stroma appeared to occur at a later stage, with a clear stepwise change evident between T3 and T4 tumours.

Although the present results are based on observational data, they potentially inform our understanding of the nature of the tumour microenvironment and its development in patients with colorectal cancer. Loss of the anti-tumour immune response, or 'immune escape' may be the potential precipitant allowing sustained tumour growth and invasion (226, 260), with other adverse tumour microenvironment characteristics occurring further downstream in the presence of "pro-tumour" local and systemic immune responses (398). Certainly, it is recognised that the immune microenvironment evolves in tandem with stage progression, favouring the development of a more pro-tumour "immunome" as T stage increases (500). As this progresses and anti-tumour immunity is degraded and

subsequently replaced by pro-tumour inflammatory responses, it may allow the development of further adverse tumour microenvironment characteristics, such as recruitment and activation of the tumour stroma and tumour-associated fibroblasts (502), and tumour cell dedifferentiation and budding (503, 504). In the present study, this would explain the relatively late increase in TSP and presence of an infiltrative margin.

Assessment of characteristics of the local and systemic environment determined survival, even after controlling for T stage. In addition, assessment of the GMS and the Immunoscore each respectively stratified survival of patients greater than nodal status. This is consistent with previous work whereby assessment of the tumour microenvironment, and in particular the immune response, may yield greater prognostic value than TNM stage itself confers (260). Much like the GMS stratifies prognosis greater than either of its determinants alone, it would be of interest to examine the clinical utility of assessment of the tumour microenvironment in its entirety; indeed it would be expected that combined assessment of the immune response, tumour-associated fibroblasts, and the tumour itself (as assessed by tumour budding) would synergistically stratify survival greater than each individual component (255, 505).

In addition to MMR status, numerous other molecular characteristics have been confirmed to hold prognostic value in patients with colorectal cancer (492, 499). Despite this, such techniques are not uniformly employed in routine clinical practice and remain costly. Therefore, it was of interest that in the present study assessment of the local and systemic environment was of greater prognostic value than MMR status. The present results further support those of Chapter 5, whereby assessment of systemic and local inflammatory profiles were shown to hold prognostic value independent of MMR status. Similarly, previous work suggests that assessment of the local environment, and in particular the inflammatory cell infiltrate, may predict survival independent of more extensive molecular characterisation (200, 201, 430). The relatively simple methodologies employed in the

present study, and their reliance on routine pathological specimens, would make them attractive candidates not only for widespread clinical use, but also for retrospective application to previously recruited clinical trials. Indeed, the relative prognostic value of comprehensive assessment of the local and systemic environment compared to comprehensive molecular characterisation remains to be fully determined. Furthermore, whether individual molecular subtypes express a phenotypical local and systemic environment would be of considerable interest.

Although tumour necrosis was strongly associated with increasing T stage consistent with previous reports (235, 506), it was not a determinant of prognosis. As the tumour grows in size, the number of tumour cells increases rapidly to a point where its supporting vasculature can no longer sustain tissue oxygen tensions and intratumoural hypoxia becomes more prevalent, resulting in unprogrammed cell death and necrosis (507). However, the development of tumour necrosis is multifactorial, and may be influenced by oncogenic pathway activation (506), the local immune response (235), and, as observed in Chapter 4, the presence of a protective expanded tumour-associated stroma. As such, any prognostic value could potentially be attributed to these numerous upstream phenomena rather than the presence of necrosis itself.

Although informative, it is clear that TNM staging is suboptimal, particularly given the lack of a stepwise increase in risk with increasing disease stage (162). Current staging, and therefore prognosis and treatment, is heavily weighted towards the presence of lymph node metastases. However, subsequent revisions of the TNM staging system have introduced significant changes to pathological definitions, particularly with respect to nodal stage and often with little supporting evidence (160). Indeed, such changes have led to concern regarding potential "upstaging of patients" without any significant implications for prognosis (408, 409). Given that the criteria for T stage remains relatively standardised and largely unchanged since first described by Dukes (154), it presents an attractive and

logical foundation to base disease staging upon. Several proposed schemes have utilised T stage as an important determinant of disease stage in combination with other factors, such as venous invasion, and with less reliance on the presence of nodal involvement as a defining factor for high-risk disease (432, 508, 509). Although the present study was largely limited to patients with T3 disease, it displays how assessment of the tumour local and systemic environment may be utilised in the routine staging of patients with primary operable colorectal cancer. However further studies, particularly encompassing patients with T1/2 disease are required to examine both the nature of the microenvironment as well as the clinical utility of such assessments across all disease stages.

The present study is limited by the small number of patients with early stage disease. Indeed, relatively few patients with T1/T2 tumours were included, and as such it was not possible to examine the prognostic value of the above measures in this patient group. Given the increasing predominance of this patient group with the advent of screening programmes (496), this would be of considerable interest. Furthermore, tumour budding was not examined in this cohort. Although strongly associated with the configuration of the infiltrative margin (510), the presence of budding is phenotypical of epithelial-mesenchymal transitioning. Given its increasing interest as an independent predictor of poor survival (511), it would be of interest to examine its prognostic utility relative to other components of the local and systemic environment.

In conclusion, the present study confirms the relationship between tumour invasiveness, as assessed by T stage, and the presence of adverse local and systemic environment characteristics, and shows how such characteristics may be utilised to guide prognosis to a greater extent than current TNM-based staging of patients with primary operable colorectal cancer.

.

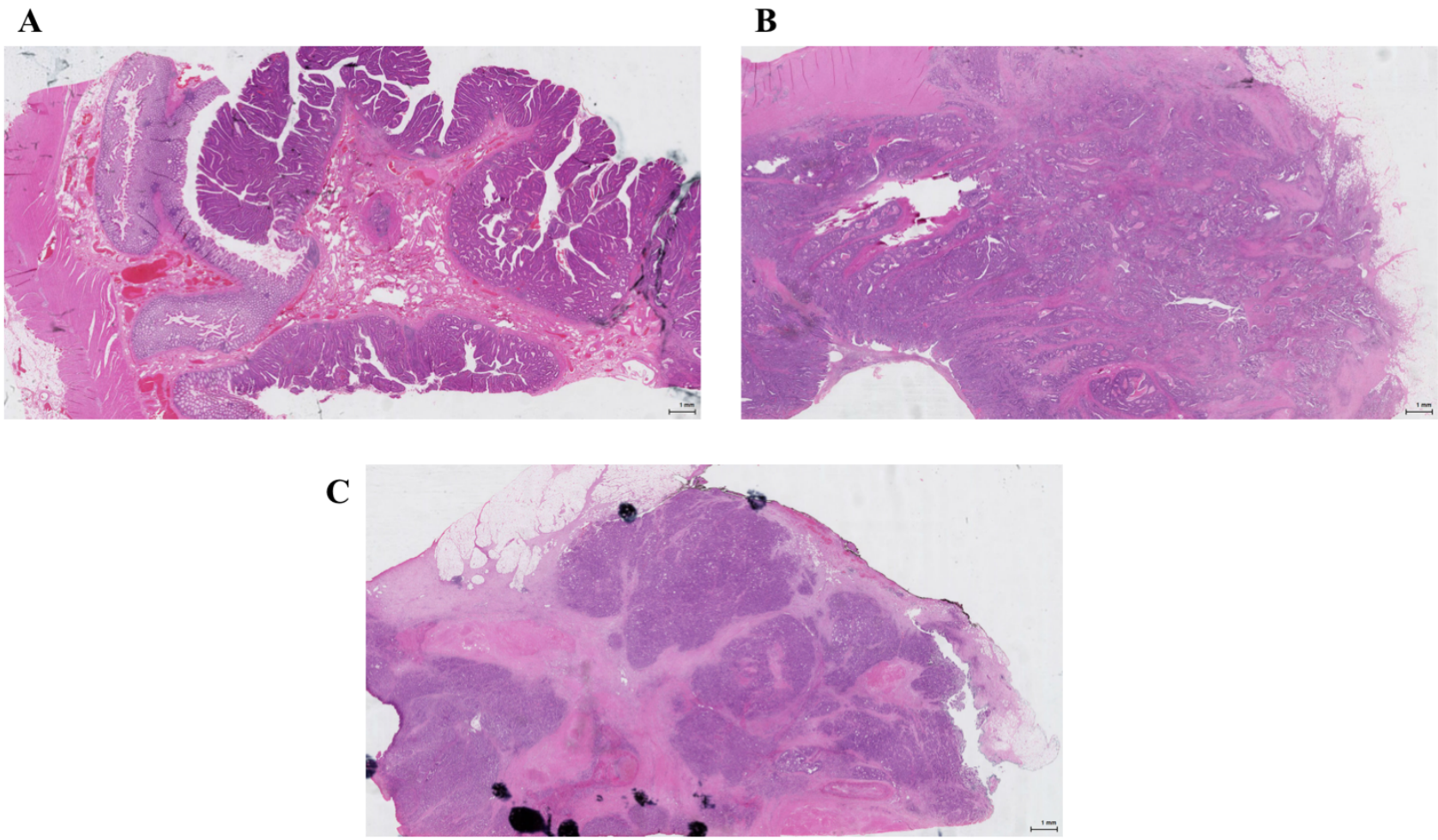


Figure 8.1 Colorectal cancer T stage (x100 magnification). **(A)** shows an example of a T1 tumour with invasion into submucosa. **(B)** shows an example of a T3 tumour with invasion through muscularis propria into surround serosal tissue without breach of peritoneum. **(C)** shows an example of a T4 tumour with invasion onto peritoneal surface

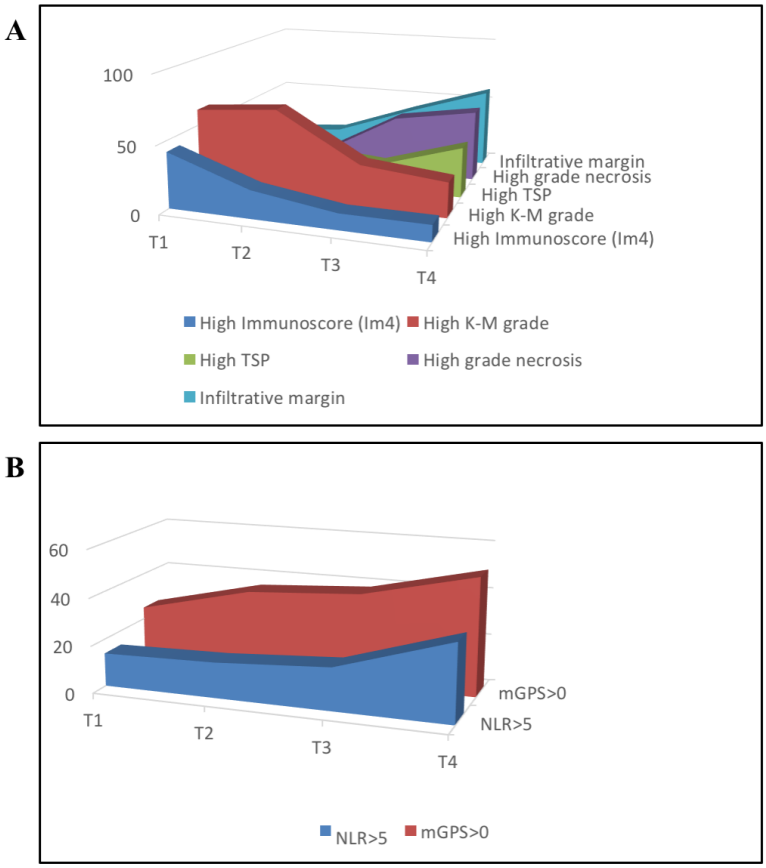
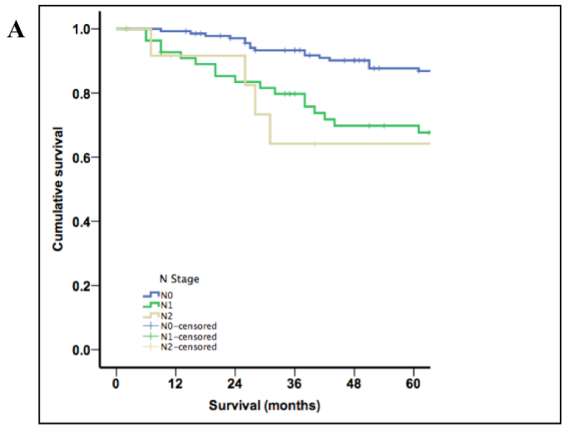
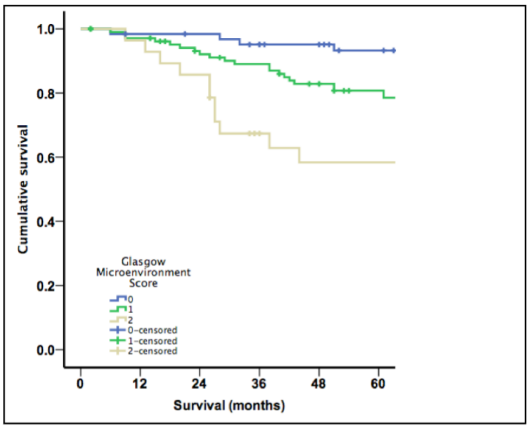


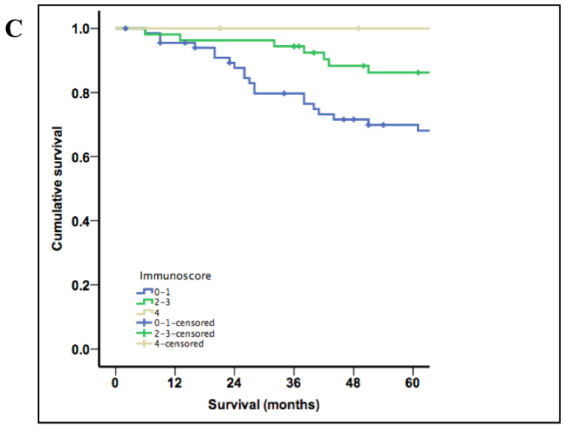
Figure 8.2 The relationship between T stage and adverse characteristics within the local and systemic environment of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer. **(A)** tumour microenvironment characteristics, and **(B)** systemic environment characteristics, The y-axis denotes the percentage of patient within each T stage with each adverse characteristic



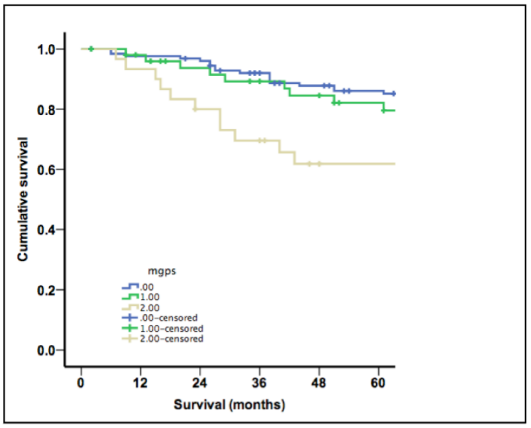
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
N0	139	138	130	122	113	104
N1	56	50	46	41	35	33
N2	13	10	10	7	6	6



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
GMS 0	63	61	60	57	54	49
GMS 1	105	100	92	87	79	73
GMS 2	28	27	24	16	13	13



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Im 0-1	68	63	56	49	43	39
Im 2-3	54	53	52	51	43	41
Im 4	17	17	16	16	16	15



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
mGPS 0	127	123	121	111	103	97
mGPS 1	51	47	42	39	36	32
mGPS 2	30	28	23	20	15	14

Figure 8.3 The relationship between pathological and local and systemic environment characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of T3 colorectal cancer. **(A)** N stage (P=0.031), **(B)** Glasgow Microenvironment Score (P<0.001), **(C)** Immunoscore (P=0.001), and **(D)** modified Glasgow Prognostic Score (P=0.004). All P-values calculated using log-rank analysis

Table 8.1 The relationship between tumour invasiveness (T stage) and clinicopathological characteristics of patients undergoing elective, primary resection of T1-T4 colorectal cancer

		T1	T2	T3	T4	
Clinicopathological characteristics (*n* when data missing)		***n*=8 (%)**	***n*=25 (%)**	***n*=208 (%)**	***n*=90 (%)**	***P***
Host characteristics						
Age						0.713
	<65	1 (13)	9 (36)	69 (33)	33 (37)	
	65-74	5 (62)	8 (32)	70 (34)	27 (30)	
	>75	2 (25)	8 (32)	69 (33)	30 (33)	
Sex						0.533
	Female	5 (62)	16 (64)	93 (45)	46 (51)	
	Male	3 (38)	9 (36)	115 (55)	44 (49)	
Adjuvant therapy (330)	**No**	6 (75)	23 (92)	159 (76)	60 (67)	0.030
	Yes	2 (25)	2 (8)	49 (24)	29 (33)	
Tumour characteristics						
Tumour site						<0.001
	Colon	2 (25)	12 (48)	145 (70)	73 (81)	
	Rectum	6 (75)	13 (52)	63 (30)	17 (19)	
N stage						0.002
	0	5 (62)	20 (80)	139 (67)	45 (50)	
	1	3 (38)	4 (16)	56 (27)	32 (36)	
	2	0 (0)	1 (4)	13 (6)	13 (14)	
Tumour differentiation						0.016
	Well/ mod	7 (87)	24 (96)	189 (91)	72 (80)	
	Poor	1 (13)	1 (4)	19 (9)	18 (20)	
Margin involvement						<0.001
	Absent	8 (100)	25 (100)	205 (99)	72 (80)	
	Present	0 (0)	0 (0)	3 (1)	18 (20)	
Venous invasion						<0.001
	Absent	8 (100)	23 (92)	140 (67)	45 (50)	
	Present	0 (0)	2 (8)	68 (33)	45 (50)	
Mismatch repair status (209)	**Competent**	7 (87)	15 (88)	110 (87)	44 (77)	0.161
	Deficient	1 (13)	2 (12)	17 (13)	13 (23)	

Data analysed using χ^2 analysis for linear trend.

Table 8.2 The relationship between tumour invasiveness (T stage), and the local and systemic environment of patients undergoing elective, primary resection of T1-T4 colorectal cancer

		T1 (%)	T2 (%)	T3 (%)	T4 (%)	
Tumour microenvironment (*n* when data missing)		***n*=8 (%)**	***n*=25 (%)**	***n*=208 (%)**	***n*=90 (%)**	***P***
Necrosis						<0.001
(297)	**Absent**	7 (87)	19 (90)	106 (56)	37 (46)	
	Present	1 (13)	2 (10)	82 (44)	43 (54)	
Invasive margin						<0.001
(312)	**Expansile**	7 (87)	18 (82)	119 (60)	34 (40)	
	Infiltrative	1 (13)	4 (18)	78 (40)	51 (60)	
Klintrup-Mäkinen						0.001
grade (307)	**Strong**	5 (62)	14 (67)	63 (32)	21 (26)	
	Weak	3 (38)	7 (33)	133 (68)	61 (74)	
Tumour stroma						0.006
percentage	**Low**	7 (87)	19 (76)	168 (81)	56 (62)	
(331)	**High**	1 (13)	6 (24)	40 (19)	34 (38)	
Immunoscore						0.016
(226)	**0-1**	2 (29)	8 (42)	68 (49)	37 (61)	
	2-3	2 (29)	7 (37)	54 (39)	17 (28)	
	4	3 (42)	4 (21)	17 (12)	7 (12)	
Systemic environment (*n* when data missing)						
Modified Glasgow						0.031
Prognostic Score	**0**	6 (75)	16 (64)	127 (61)	45 (51)	
(330**)**	**1**	2 (25)	8 (32)	51 (25)	29 (33)	
	2	0 (0)	1 (4)	30 (14)	15 (17)	
Neutrophil:						0.033
lymphocyte ratio	**≤5**	6 (86)	17 (85)	115 (82)	39 (67)	
(225)	**>5**	1 (14)	3 (15)	25 (18)	19 (33)	

Data analysed using χ^2 analysis for linear trend.

Table 8.3 The interrelationship between tumour microenvironment and systemic environment characteristics of patients undergoing elective, primary resection of T1-T4 colorectal cancer

	Necrosis	**Invasive margin**	**KM grade**	**TSP**	**Immunoscore**	**mGPS**
Invasive margin	0.774	-	-	-	-	-
KM grade	0.142	**<0.001**$^{-}$	-	-	-	-
TSP	**0.001**$^{-}$	**<0.001**$^{+}$	**0.069**$^{-}$	-	-	-
Immunoscore	**0.014**$^{-}$	0.185	**<0.001**$^{+}$	0.569	-	-
mGPS	**<0.001**$^{+}$	0.593	0.465	0.177	0.189	-
NLR	**0.021**$^{+}$	0.298	**0.076**$^{-}$	0.558	0.562	**<0.001**$^{+}$

Displayed values are *P*-values calculated using χ^2 test for linear trend between variables.
$^{+}$ positive correlation, $^{-}$ inverse correlation

Table 8.4 The relationship between T stage, clinicopathological and local and systemic environment characteristics and five-year cancer-specific survival of patients undergoing elective, primary resection of T1-T4 colorectal cancer

		All (T1-T4)			T3 disease		
		N	**5-yr CSS % (SE)**	***P***	***N***	**5-yr CSS % (SE)**	***P***
All		331	77 (2)	-	208	82 (3)	-
N stage				<0.001			0.031
	N0	209	86 (2)		139	88 (3)	
	N1	95	64 (5)		56	70 (6)	
	N2	27	46 (10)		13	64 (14)	
Mismatch repair status				0.100			0.206
	Deficient	33	88 (6)		17	94 (6)	
	Competent	176	73 (3)		110	79 (4)	
Necrosis				0.130			0.404
	Absent	169	80 (3)		106	84 (4)	
	Present	128	72 (4)		82	77 (5)	
Invasive Margin				<0.001			0.004
	Expansile	178	82 (3)		119	85 (3)	
	Infiltrative	134	69 (4)		78	75 (5)	
Klintrup-Mäkinen grade				<0.001			0.001
	Strong	103	90 (3)		63	93 (3)	
	Weak	204	70 (3)		133	76 (4)	
Tumour stroma percentage				<0.001			0.018
	Low	250	81 (3)		168	84 (3)	
	High	81	64 (6)		40	71 (7)	
Glasgow Microenvironment Score				<0.001			<0.001
	0	103	90 (3)		63	93 (3)	
	1	147	77 (4)		105	81 (4)	
	2	57	53 (7)		28	58 (10)	
Immunoscore				<0.001			0.001
	4	31	96 (3)		17	100 (0)	
	2-3	80	87 (4)		54	87 (5)	
	0-1	115	62 (5)		68	70 (6)	
Modified Glasgow Prognostic Score				<0.001			0.004
	0	294	83 (3)		127	86 (3)	
	1	90	72 (5)		51	82 (6)	
	2	46	57 (8)		30	62 (9)	
Neutrophil: Lymphocyte Ratio				0.362			0.091
	≤5	177	79 (3)		115	84 (3)	
	>5	48	73 (7)		25	74 (9)	

Log-rank *P*-value provided for the prognostic value of individual characteristics. CSS – cancer-specific survival, SE – standard error.

.

9 Comparison of the prognostic value of measures of the tumour inflammatory cell infiltrate and tumour-associated stroma in patients with primary operable colorectal cancer

9.1 Introduction

Although semi-quantitative assessment of the generalised inflammatory cell infiltrate, such as that offered by the KM grade, has been validated as a stage-independent prognostic characteristic (248, 249), the prognostic value of immunohistochemistry-based assessments of immune cell type and location within the tumour microenvironment is of interest (232, 501). Initially describing the density of cytotoxic ($CD8^+$) and memory ($CD45R0^+$) T-lymphocytes within the tumour microenvironment, the Immunoscore has recently been refined to reflect a cumulative score based on the density of the overall mature $CD3^+$ T-lymphocyte population in addition to the $CD8^+$ T-lymphocyte population at the invasive margin and within the tumour core, and has been validated as a prognostic marker with superior prognostic ability when compared to TNM staging in colorectal cancer (260, 501, 512). However, whether the Immunoscore, with all the inherent complexities of immunohistochemistry, is superior to the KM grade remains to be determined.

In Chapter 7, a novel, cumulative tumour microenvironment-based score, comprised of KM grade and TSP, was shown to stratify survival greater than either measure alone in patients undergoing elective, potentially curative resection of primary operable colorectal cancer. Using this prognostic score termed the Glasgow Microenvironment Score (GMS) it was possible to further stratify five-year cancer-specific survival of those patients with a weak local inflammatory cell infiltrate from 75% to 51%. Indeed, the GMS has much to commend it, given its reliance on routinely available pathological specimens and rapid, reproducible, semi-quantitative histopathological assessments. However, whether inclusion of a potentially more detailed measure of the inflammatory cell infiltrate, such as the Immunoscore, may alter the prognostic value of the GMS and the tumour-associated

stroma in particular is not clear. Therefore, the present study had two aims: first, to compare the prognostic value of assessment of the inflammatory cell infiltrate using the Klintrup-Mäkinen grade and the Immunoscore, and second, to examine the clinical utility of combined assessment of the inflammatory cell infiltrate and TSP.

9.2 Patients and Methods

Clinicopathological characteristics

Patients were identified from a prospectively collected and maintained database of elective and emergency colorectal cancer resections performed in a single surgical unit at GRI. For the purposes of the present chapter, patients who had undergone emergency or elective, potentially curative resection of stage I-III colorectal adenocarcinoma without neoadjuvant therapy were included. Other inclusion and exclusion criteria, clinicopathological staging, multi-disciplinary team review and follow-up protocols has previously been described in Chapter 2 and 4. Assessment of MMR status was performed as described in Chapter 5.

Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st March 2014 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from recurrent or metastatic colorectal cancer.

Assessment of the tumour microenvironment

The KM grade and TSP were both assessed using routine H&E-stained sections of the deepest point of tumour invasion as previously described in Chapter 4. The KM grade was categorised as low grade or high grade, and TSP was categorised as low (≤50%) or high (>50%).

Full sections of the invasive margin were stained for mature T-lymphocytes ($CD3^+$) and cytotoxic ($CD8^+$) T-lymphocytes as previously described in Chapter 4. The Immunoscore was calculated as described in Chapter 8. Briefly, the density of $CD3^+$ and $CD8^+$ T-cells within the invasive margin and tumour centre were separately semi-quantitatively graded as high or low; and the Immunoscore was calculated from the number of regions with a high $CD3^+$ and $CD8^+$ cell density, giving five potential groups (Im0, Im1, Im2, Im3, Im4),

ranging from all regions low density (Im0) to all regions high density (Im4). An example of different $CD3^+$ T-lymphocyte densities within different tumour microenvironment regions is displayed in Figure 9.1.

Statistical analysis

The relationship between components of the tumour microenvironment and cancer-specific survival was examined using Kaplan-Meier log-rank analysis, with five-year survival presented as percentage surviving (SE). The relationship between components of the tumour microenvironment, clinicopathological characteristics and survival was examined using multivariate Cox proportional hazards regression to calculate HRs and 95% CIs. Variables with a *P*-value ≤0.05 on univariate regression analysis were entered into a multivariate model using a backward conditional method. All statistical analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). A *P*-value ≤0.05 was considered statistically significant.

9.3 Results

The study population was comprised of 246 patients undergoing potentially curative resection of stage I-III colorectal cancer. Clinicopathological characteristics are displayed in Table 9.1. Approximately two thirds of patients were 65 years of age or older at time of surgery and 52% were male. Fifteen patients (6%) underwent emergency resection, and just over two thirds of patients underwent resection of colon cancer. Histopathological reporting confirmed stage I, stage II and stage III disease in 7%, 52% and 41% of patients respectively. Mismatch repair status was available for 205 patients; 30 patients (15%) had mismatch repair deficient colorectal cancer.

The median follow-up of survivors was 145 months (range 87-206 months) with 76 colorectal cancer-related deaths and 76 non-cancer deaths. Five-year survival was 74% for cancer-specific survival and 63% for overall survival. In total, 71 patients (29%) received adjuvant chemotherapy; one patient with stage I (6%) disease, 19 patients with stage II disease (15%), and 51 patients with stage III disease (51%) received adjuvant chemotherapy.

The relationship between the local inflammatory cell infiltrate and cancer-specific survival

The relationship between measures of the local inflammatory cell infiltrate and cancer-specific survival is displayed in Figure 9.2 and Table 9.2. A low KM grade was associated with poorer five-year survival (66% vs. 88%; P=0.002). When stratified by tumour site, low KM grade was associated with poorer survival of patients with colon cancer (P=0.018) and showed a trend towards poorer survival of patients with rectal cancer (P=0.068). When stratified by TNM stage, low KM grade showed a trend towards poorer survival of patients with node negative (stage I/II) disease (P=0.053) and node positive (stage III) disease (P=0.057). Finally, low KM grade was associated with poorer survival of both

patients who received and did not receive adjuvant therapy (P=0.046 and P=0.028 respectively).

The relationship between Immunoscore and cancer-specific survival is displayed in Figure 9.2 and Table 9.2. Five-year cancer-specific survival ranged from 93% for patients with Im4 to 61% for patients with Im0 (P<0.001). The survival of patients with Im0 and Im1, or Im2 and Im3 did not differ significantly (P=0.788 and P=0.599, respectively). As such, for further statistical analysis, the Immunoscore was refined to stratify patients in to three prognostic groups: Im4, with five-year survival of 93%; Im2/3, with five-year survival of 84%; and Im0/1, five-year survival of 61% (P<0.001). When stratified by tumour site, a low Immunoscore was associated with poorer survival of patients with both colon and rectal cancer (P=0.003 and P=0.001 respectively). When stratified by TNM stage, low Immunoscore was associated with poorer survival of patients with stage I/II disease (P=0.002) and stage III disease (P=0.011). Finally, low Immunoscore was associated with poorer survival of patients who did not receive adjuvant chemotherapy (P<0.001) and showed a trend towards poorer survival in patients who did receive adjuvant therapy (P=0.059).

Klintrup-Mäkinen grade was strongly associated with the Immunoscore (P<0.001; Figure 9.3). Comparison between the prognostic value of the KM grade and Immunoscore was subsequently performed (Table 9.3). The Immunoscore was able to further stratify the survival of patients with both a low and high KM grade; the survival of patients with a low KM grade ranged from 90% (Im4) to 60% (Im0/1) (P=0.015), whereas the survival of patients with a high KM grade ranged from 94% (Im4) to 71% (Im0/1) (P=0.010). In contrast, KM grade did not further significantly stratify the Immunoscore.

The relationship between tumour stroma percentage, the tumour inflammatory cell infiltrate and cancer-specific survival

The prognostic value of combined assessment of the inflammatory cell infiltrate and TSP was subsequently examined (Table 9.4). Tumour stroma percentage significantly stratified the survival of patients from 80% (low TSP) to 57% (high TSP) (P=0.001). In combination with assessment of the inflammatory cell infiltrate, TSP significantly stratified survival of those with a weak infiltrate but not those with a strong infiltrate. In particular, TSP significantly stratified survival of patients with a low KM grade from 75% to 47% (P<0.001), whereas in patients with a high KM grade, survival of patients with a low TSP was comparable to that of patients with a high TSP (P=0.485). In combination with the Immunoscore, the effect of TSP on survival decreased as the Immunoscore increased; TSP stratified the survival of patients with Im0/1 from 71% to 38% (P<0.001) and patients with Im2/3 from 87% to 77% (P=0.069), but not patients with Im4 (P=0.545) (Figure 9.4). Conversely, assessment of the inflammatory cell infiltrate was able to stratify survival of patients with both a high and low TSP; KM grade stratified patients with a low TSP from 88% to 75% (P=0.081) and patients with a high TSP from 87% to 47% (P=0.034), whereas Immunoscore stratified survival of patients with a low TSP from 92% to 71% (P=0.002) and patients with a high TSP from 100% to 38% (P=0.004).

The relationship between Klintrup-Mäkinen grade, Immunoscore, tumour stroma percentage, clinicopathological characteristics and cancer-specific survival

On univariate survival analysis (Table 9.5), emergency presentation, T stage, mGPS (both P<0.05), N stage, venous invasion, margin involvement and peritoneal involvement (all P≤0.001) were associated with cancer-specific survival. The KM grade (P=0.003), Immunoscore and TSP were all associated with survival (both P<0.001).

On multivariate analysis, after controlling for age, sex, tumour site and adjuvant therapy and considering all variables significant on univariate analysis (Model 1), the

Immunoscore and TSP (both $P<0.01$), but not the KM grade, were associated with survival independent of venous invasion ($P=0.001$) and mGPS ($P<0.05$). When the Immunoscore was removed from the multivariable model (Model 2), KM grade ($P<0.05$) and TSP ($P<0.01$) remained associated with survival independent of venous invasion ($P=0.001$) and mGPS ($P<0.01$).

9.4 Discussion

In the present study, an immunohistochemistry-based assessment of the inflammatory cell infiltrate was superior to that of H&E-based assessment in predicting outcome of patients undergoing potentially curative resection of stage I-III colorectal cancer. Furthermore, the combination of assessment of the inflammatory cell infiltrate, using either KM grade or Immunoscore, and the tumour-associated stroma, using TSP, provided additional prognostic information.

The present study compared the prognostic utility of two validated measures of the tumour inflammatory cell infiltrate – the KM grade and the Immunoscore (248, 501). Although both were associated with cancer-specific survival, the Immunoscore, an immunohistochemistry-based assessment of $CD3^+$ and $CD8^+$ T-lymphocyte density, was able to stratify survival of patients to a greater degree than KM grade, an H&E-based assessment of the generalised inflammatory cell infiltrate. In particular, the Immunoscore was able to stratify survival of patients with both a low and high KM grade; indeed, survival of patients with a low KM grade but high Immunoscore was comparable to that of patients with a high KM grade.

The relative difference in the prognostic value of both measures of the inflammatory cell infiltrate may be explained by the components of the immune response that each measures. Whereas KM grade provides a measure of the overall, generalised inflammatory cell infiltrate, the Immunoscore measures the host adaptive T-lymphocytic response to cancer. Indeed, although an increase in KM grade is associated with an increase in the density of tumour-infiltrating T-lymphocytes (233, 433, 452), it is also associated with an increase in the density of the innate immune infiltrate, and in particular neutrophils and macrophages (233, 452). In the present study, within the group of patients with a high KM grade, the number of patients with a low (Im0/1, n=19) or high (Im4, n=21) Immunoscore (as a

measure of adaptive immunity) was similar, whereas of those patients with a low KM grade, a small number had a high Immunoscore. Therefore the KM grade may not always represent the same entity. However, although the importance of host adaptive anti-tumour immune responses is recognised, it is now appreciated that myeloid-derived cells, such as neutrophils and macrophages, play an important functional role in promoting tumour progression (513). Indeed, it remains to be determined whether immunohistochemistry-based assessment of the innate immune infiltrate may increase the clinical and prognostic utility of measuring the inflammatory cell infiltrate in patients with colorectal cancer.

It was of interest in the present cohort that TSP, an assessment of the tumour-associated stroma, was associated with survival independent of either measure of the inflammatory cell infiltrate, and that combined assessment provided greater prognostic value. For example, it was possible to stratify five-year survival from 92% (Im4, low TSP) to 38% (Im0/1, high TSP). Furthermore, although the relationship between TSP and survival was strongest in patients with a poor inflammatory cell infiltrate, both the number of patients with a high TSP, and its prognostic value, decreased as the density of the inflammatory infiltrate increased. Although it has previously been suggested that the presence of a tumour-associated stroma precludes effective infiltration of the tumour microenvironment by an anti-tumour immune response (443), the present results are consistent with those of Chapter 4 and 7, and may favour the alternative hypothesis that loss of the adaptive immune infiltrate predisposes to the development of a pro-tumour stromal compartment, potentially mediated by the residual innate immune infiltrate (502).

Consistent with the GMS proposed in Chapter 7, the present results suggest that a similar scheme may be applied to the combination of the Immunoscore and TSP and may have even greater clinical utility. Indeed, such a combination may optimise risk prediction in patients undergoing colorectal cancer resection by identifying both those with an excellent prognosis (Im4; five-year cancer-specific survival of 93%), and those with an extremely

poor prognosis who may benefit from adjuvant therapy (Im0/1, high TSP; five-year cancer-specific survival of 38%).

In the present study, it was of interest that both the systemic inflammatory response as measured by mGPS, and the local inflammatory cell infiltrate as measured by either KM grade or the Immunoscore had independent prognostic value on multivariate analysis. It is likely that these measures reflect different aspects of the same underlying process, and therefore it would be of interest to compare the local and systemic inflammatory responses and how they may be combined to form a prognostic score. Indeed, Turner and colleagues have recently combined measures of the local and systemic inflammatory response to give better risk stratification in patients with node negative colorectal cancer (514). However, the rationale of their approach that combined the NLR and assessment of the chronic inflammatory cell density was not clear, since different cell types were assessed locally and systemically. Indeed, only approximately 20% of patients had an elevated NLR and a low chronic inflammatory cell density, and therefore this score does not capture the same entity. Similarly, combinations of other systemic and local inflammatory measures, such as the mGPS and KM grade or Immunoscore, will have such limitations. Moreover, the numbers of patients included in the present analysis limits the value of such analysis and therefore was not formally examined.

The present analysis is perhaps limited by its use of manual, semi-quantitative assessment of the inflammatory cell infiltrate as opposed to automated assessment as has been recommended for routine assessment of the Immunoscore (501). However, the manual techniques employed showed excellent inter-operator agreement (249, 433) and manual assessment of the inflammatory cell infiltrate has been shown to correlate strongly with automated assessment (445, 464). Furthermore, manual assessment of immunohistochemical staining may allow for greater discrimination of non-specific, background staining and provide superior prognostic value compared to automated

assessment (445). Furthermore, meaningful statistical analysis was precluded by the small number of patients in particular subgroups, such as those with stage II disease and high-risk pathological characteristics, or patients with stage I disease. Finally, the results of the present study, and in particular the prognostic utility of combined assessment of the inflammatory cell infiltrate and tumour-associated stroma, remain to be validated in an independent patient cohort from an independent centre.

In conclusion, the present results suggest that the prognostic value of an immunohistochemistry-based assessment of the inflammatory cell infiltrate is superior to H&E-based assessment in patients undergoing potentially curative resection of stage I-III colorectal cancer. Furthermore, TSP improves the prediction of survival by either measure of the inflammatory cell infiltrate.

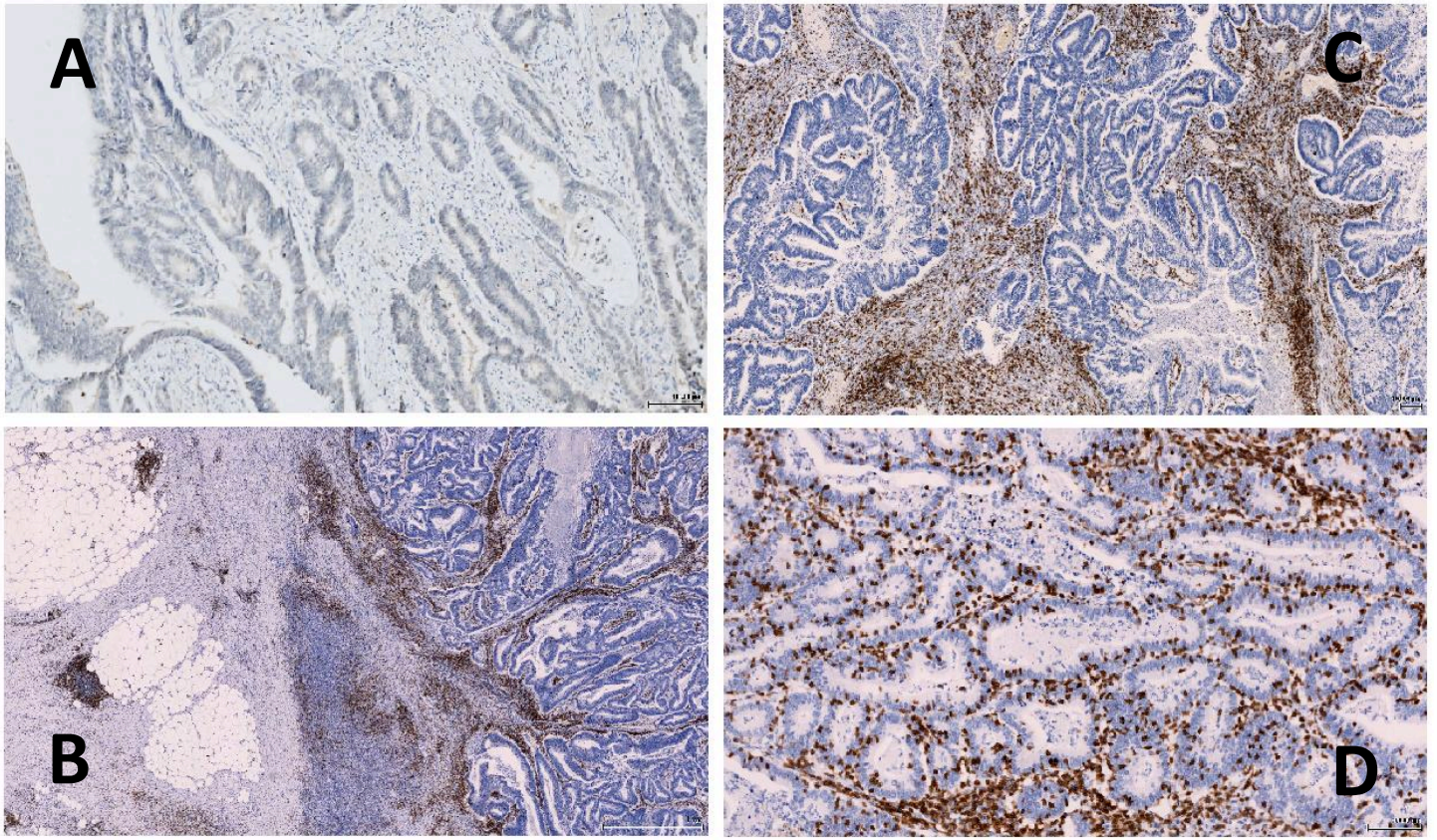
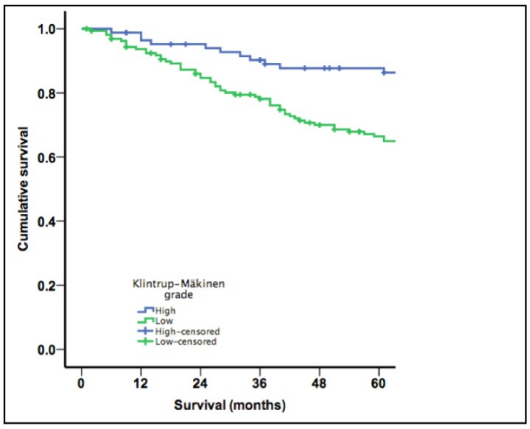


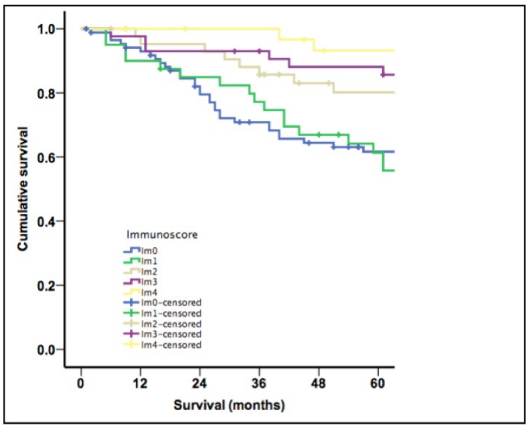
Figure 9.1 Examples of $CD3^+$ T-lymphocyte staining in patients with colorectal cancer (x200 magnification). **(A)** displays low density, whereas **(B)** displays a high density at the invasive margin. **(C)** displays a high stromal density and **(D)** displays a high intraepithelial/ cancer cell nest density of $CD3^+$T-lymphocytes

A



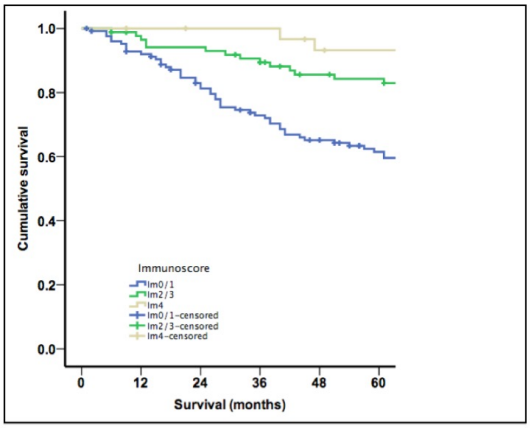
Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low grade	161	147	132	118	101	91
High grade	85	82	77	73	67	64

B



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Im0	87	79	66	55	49	43
Im1	40	36	33	30	26	22
Im2	44	41	4	37	30	28
Im3	43	42	40	39	36	36
Im4	32	31	30	30	27	26

C



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Im0/1	127	115	99	85	75	65
Im2/3	87	83	80	76	66	64
Im4	32	31	30	30	27	26

Figure 9.2 The relationship between the local inflammatory cell infiltrate and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer. **(A)** Klintrup-Mäkinen grade (P=0.002), **(B)** Immunoscore (P<0.001), and **(C)** Immunoscore groups (P<0.001). All P-values calculated using log-rank analysis

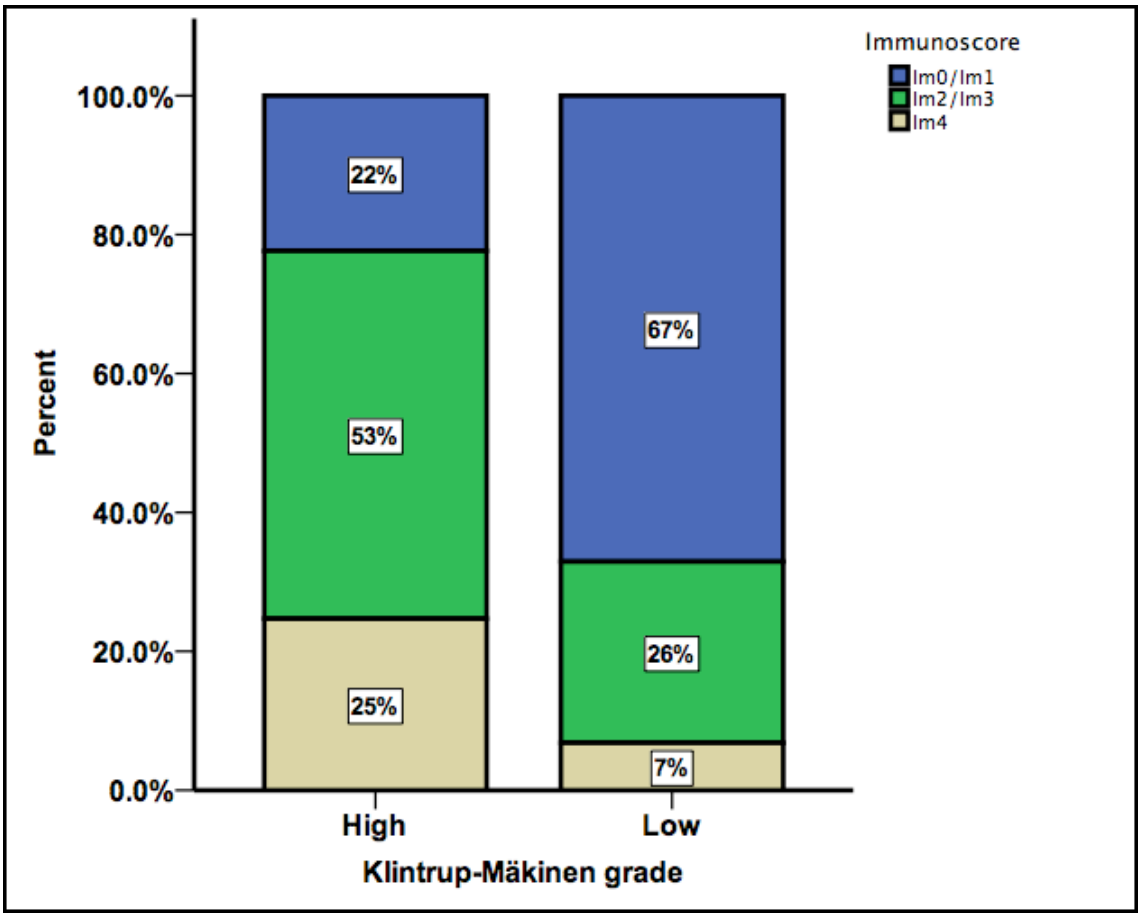
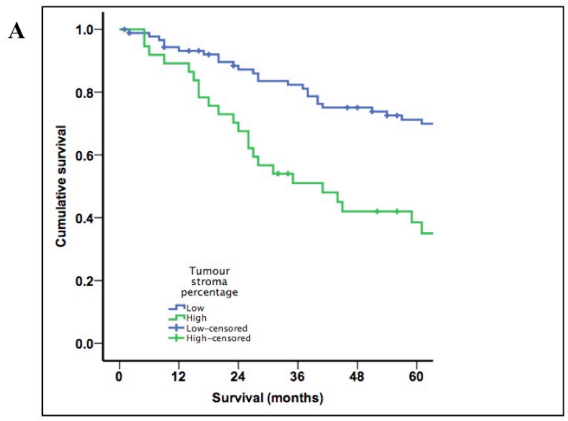
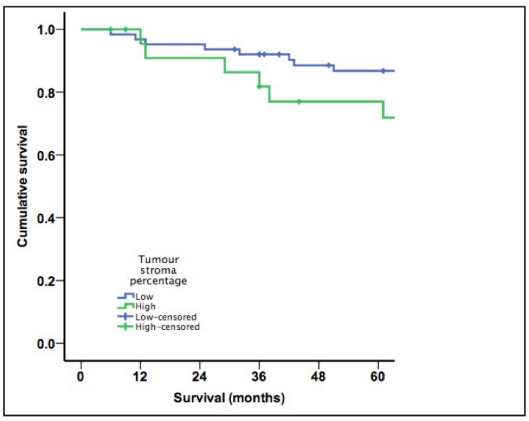


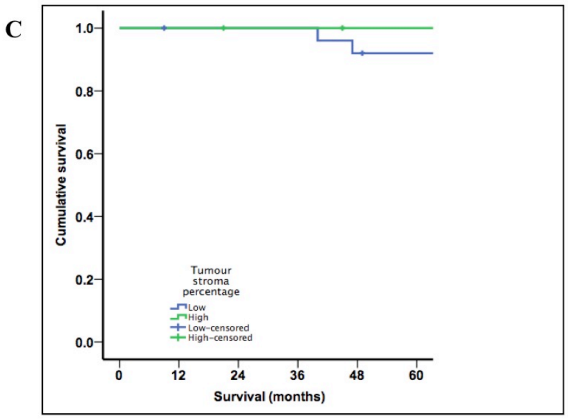
Figure 9.3 The relationship between Klintrup-Mäkinen grade and Immunoscore in patients undergoing potentially curative resection of stage I-III colorectal cancer (χ^2 analysis for linear trend P<0.001)



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	90	82	73	68	61	54
High TSP	37	33	26	17	14	11



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	63	61	60	57	51	49
High TSP	24	22	20	19	15	15



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low TSP	26	25	25	25	23	22
High TSP	6	6	5	5	4	4

Figure 9.4 The relationship between Immunoscore, tumour stroma percentage and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer. **(A)** Im0/1 (P<0.001), **(B)** Im2/3 (P=0.069), and **(C)** Im4 (P=0.545). All P-values calculated using log-rank analysis

Table 9.1 Clinicopathological characteristics of patients undergoing potentially curative resection of stage I-III colorectal cancer

		All
Clinicopathological Characteristics (*n* when data missing)		***n*=246 (%)**
Host characteristics		
Age		
	<65	82 (33)
	65-74	84 (34)
	>75	80 (33)
Sex		
	Female	117 (48)
	Male	129 (52)
Modified Glasgow Prognostic Score		
	0	138 (56)
	1	80 (33)
	2	28 (11)
Presentation		
	Elective	231 (94)
	Emergency	15 (6)
Adjuvant therapy		
	No	175 (51)
	Yes	71 (29)
Tumour characteristics		
Tumour site		
	Colon	169 (69)
	Rectum	77 (31)
TNM stage		
	I	18 (7)
	II	128 (52)
	III	100 (41)
T stage		
	1-2	26 (11)
	3	152 (62)
	4	68 (28)
N stage		
	0	146 (59)
	1	77 (31)
	2	23 (9)
Lymph nodes examined		
	<12	159 (65)
	≥12	87 (35)
Tumour differentiation		
	Mod/well	216 (88)
	Poor	30 (12)
Venous invasion		
	No	158 (64)
	Yes	88 (36)
Margin involvement		
	No	230 (94)
	Yes	16 (7)
Peritoneal involvement		
	No	178 (72)
	Yes	68 (28)
Tumour perforation		
	No	238 (97)
	Yes	8 (3)
Mismatch repair status (205)	**Competent**	175 (85)
	Deficient	30 (15)

Table 9.2 Comparison between measures of the local inflammatory cell infiltrate, tumour stroma percentage and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer

			Tumour site				TNM stage				Adjuvant therapy			
	All patients		Colon		Rectum		TNM I/ II		TNM III		No		Yes	
	N	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)	*N*	5-yr CSS % (SE)
Klintrup-Mäkinen grade														
Low grade	161	66 (4)	111	65% (5)	50	69% (7)	89	77% (5)	72	53% (6)	110	69% (5)	51	61% (7)
High grade	85	88 (4) 0.002	58	88% (4) 0.018	27	88% (6) 0.068	57	93% (4) 0.053	28	78% (8) 0.057	65	87% (4) 0.028	20	89% (7) 0.046
Immunoscore														
Im0/1	127	61 (4)	87	63% (5)	40	58% (8)	69	72% (6)	58	49% (7)	91	62% (5)	36	59% (8)
Im2/3	87	84 (4)	60	81% (5)	27	92% (5)	54	92% (4)	33	72% (8)	60	89% (4)	27	74% (8)
Im4	32	93 (5) <0.001	22	90% (7) 0.003	10	100% (0) 0.001	23	95% (5) 0.002	9	88% (12) 0.011	24	91% (6) <0.001	8	100% (0) 0.059

Log-rank *P*-value provided for the prognostic value of KM grade and Immunoscore within each column group. CSS – cancer-specific survival, SE – standard error.

Table 9.3 The relationship between Klintrup-Mäkinen grade, Immunoscore and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer

	All (Low and high KM grade)		Low Klintrup-Mäkinen grade		High Klintrup-Mäkinen grade	
Immunoscore	**N**	**5-yr CSS % (SE)**	***N***	**5-yr CSS % (SE)**	***N***	**5-yr CSS % (SE)**
Im0/1	127	61% (4)	108	60% (5)	19	71% (11) $^{0.598}$
Im2/3	87	84% (4)	42	77% (7)	45	91% (4) $^{0.279}$
Im4	32	93% (5) $^{<0.001}$	11	90% (9) $^{0.015}$	21	94% (5) $^{0.645/0.010}$
All (Im0-4)	246	74% (3)	161	66% (4)	85	88% (4) $^{0.002}$

Log-rank *P*-value provided for the prognostic value for KM grade within each Immunoscore group (row) and for Immunoscore within each KM group (column). CSS – cancer-specific survival, SE –standard error.

Table 9.4 Comparison of the combined prognostic value of different measures of the local inflammatory cell infiltrate and tumour stroma percentage in patients undergoing potentially curative resection of stage I-III colorectal cancer

	Klintrup-Mäkinen grade						Immunoscore							
	All		Low grade		High grade		All		Im0/1		Im2/3		Im4	
TSP	***N***	**5-yr CSS % (SE)**	***N***	**5-yr CSS % (SE)**	***N***	**5-yr CSS % (SE)**	**N**	**5-yr CSS % (SE)**	**N**	**5-yr CSS % (SE)**	**N**	**5-yr CSS % (SE)**	***N***	**5-yr CSS % (SE)**
High	67	57% (6)	50	47% (7)	17	87% (9) [0.081]	67	57% (6) [b]	37	38% (8)	24	77% (9)	6	100% (0) [0.002]
Low	179	80% (3) [<0.001]	111	75% (4) [<0.001]	68	88% (4) [0.034/0.485]	179	80% (3) [<0.001]	90	71% (5) [<0.001]	63	87% (4) [0.069]	26	92% (5) [0.004/0.545]
All	246	74% (3)	161	66% (4)	85	88% (4)	246	74% (3)	127	61% (4) [c]	87	84% (4)	32	93% (5)

Log-rank *P*-value provided for the prognostic value of KM grade and Immunoscore within each TSP group (row) and TSP within each KM grade and Immunoscore group (column). CSS – cancer-specific survival, SE –standard error.

Table 9.4 displays the relationship between measures of the local inflammatory cell infiltrate (KM grade and Immunoscore), TSP and five-year cancer-specific survival of patients undergoing elective resection of stage I-III colorectal cancer. The TSP provided further prognostic stratification of patients with a low density inflammatory cell infiltrate but not a high density inflammatory cell infiltrate as measured by both KM grade and Immunoscore. Conversely, the inflammatory cell infiltrate was able to further stratify survival or patients with both a low and high TSP.

Table 9.5 The relationship between Klintrup-Mäkinen grade, Immunoscore, tumour stroma percentage, clinicopathological characteristics and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer

	Cancer-specific survival					
	Univariate HR (95% CI)	***P***	**Multivariate HR (95%CI) (Model 1)**	***P***	**Multivariate HR (95% CI) (Model 2)**	***P***
Age (<65/ 65-74/ >75)	1.18 (0.90-1.57)	0.237	-	0.444	-	0.091
Sex (female/ male)	0.93 (0.59-1.46)	0.762	-	0.065	-	0.308
Presentation (elective/ emergency)	2.22 (1.06-4.62)	0.034	-	0.724	-	0.369
Adjuvant therapy (no/ yes)	1.40 (0.88-2.24)	0.160	-	0.988	-	0.505
mGPS (0/ 1/ 2)	1.50 (1.10-2.05)	0.010	1.52 (1.09-2.11)	0.013	1.61 (1.16-2.24)	0.005
Tumour site (colon/ rectum)	0.82 (0.49-1.36)	0.433	-	0.479	-	0.316
T stage (1-2/ 3/ 4)	1.49 (1.07-2.07)	0.017	-	0.704	-	0.981
N stage (0/ 1/ 2)	1.78 (1.32-2.41)	<0.001	-	0.148	-	0.066
Lymph nodes examined (>12/ <12)	1.38 (0.87-2.17)	0.171	-	-	-	-
Differentiation (mod-well/ poor)	1.40 (0.72-2.72)	0.322	-	-	-	-
Venous invasion (no/ yes)	2.95 (1.87-4.66)	<0.001	2.20 (1.37-3.54)	0.001	2.35 (1.45-3.80)	0.001
Margin involvement (no/ yes)	3.15 (1.56-6.33)	0.001	-	0.067	-	0.096
Peritoneal involvement (no/ yes)	2.19 (1.38-3.46)	0.001	-	0.225	-	0.125
Tumour perforation (no/ yes)	2.52 (0.92-6.93)	0.072	-	-	-	0.060
MMR status (competent/ deficient)	0.42 (0.17-1.05)	0.064	-	-	-	-
Tumour stroma percentage (low/ high)	2.46 (1.56-3.89)	<0.001	2.36 (1.44-3.84)	0.001	2.05 (1.28-3.30)	0.003
Klintrup-Mäkinen grade (weak/ strong)	0.44 (0.25-0.76)	0.003	-	0.469	0.50 (0.29-0.87)	0.015
Immunoscore (Im0-1/ Im2-3/ Im4)	0.66 (0.56-0.80)	<0.001	0.43 (0.28-0.66)	<0.001		

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis to calculate two models; Model 1 using all variable significant on univariate analysis, and Model 2, excluding Immunoscore and using KM grade only.

10 The relationship between pre-operative aspirin and statin use and systemic inflammatory responses of patients undergoing potentially curative resection of colorectal cancer

10.1 Introduction

In addition to identifying patients at increased risk of recurrence following potentially curative resection of colorectal cancer, assessment of systemic inflammatory responses may also guide the use of adjuvant systemic therapies. Although an elevated mGPS suggested a lack of response to 5-FU-based chemotherapy in Chapters 2 and 3, it may however predict increased response to other, novel therapies, and in particular those targeting tumour-associated inflammatory responses. For example, recent clinical trial data has identified an elevated mGPS as a biomarker of response to ruxolitinib, a JAK inhibitor, in patients with metastatic pancreatic cancer (491).

Two potential therapeutic agents that may target tumour-associated inflammation in patients with colorectal cancer are aspirin and statins. There has been considerable interest regarding the role of aspirin in the chemoprevention of colorectal neoplasia (70, 277, 515-519), as well as as an adjunctive therapy following a diagnosis of colorectal cancer (279, 296, 520, 521). Similarly, regular statin use may have both chemopreventive and secondary prevention benefits in patients with colorectal cancer (522-524).

It is likely that the anti-neoplastic effects of both aspirin and statins reflect the pleiotropic nature of these drugs, however, both are recognised as having intrinsic anti-inflammatory properties. Indeed, pro-inflammatory and immune pathways and mediators, such as tumour COX-2 expression (521), human leukocyte antigen-1 (HLA-1) (525), and the phosphatidylinositol 3-kinase (PI3K) pathway (208, 521, 526), have all been suggested as potential therapeutic targets mediating the effects of aspirin in patients with cancer.

In a previous cohort study, elevated levels of serum sTNFR-1, but not C-reactive protein (CRP), predicted increased effect of long-term aspirin therapy in reducing colorectal cancer risk (64), whereas a further cohort study suggested that aspirin may prevent longitudinal incremental increases in serum CRP. However, this did not appear to account for its chemotherapeutic benefit in reducing the risk of subsequent colorectal adenomata (332). The relationship between aspirin and statin use and systemic inflammatory responses in patients with established colorectal cancer however remains to be determined. As such, the aim of the present study was to examine the relationship between routine use of aspirin and statins and systemic inflammatory responses in patients undergoing potentially curative resection of colorectal cancer.

10.2 Patients and Methods

Patients were identified from a prospectively collected database of colorectal cancer resections performed since January 1997 in a single surgical unit in Glasgow Royal Infirmary. For the purposes of the present study, patients who had undergone elective or emergency resection of stage I-III colorectal adenocarcinoma with curative intent from January 2010 to July 2014, and for whom pre-operative prescribing data was available, were included. Resection was deemed curative on the basis of pre-operative computed tomography and intraoperative findings. Patients who underwent local resection, resection with palliative intent or for whom pre-operative prescription data was not available, were excluded. Clinicopathological staging was performed using TNM 5th edition, and MDT review, provision of adjuvant chemotherapy and routine follow-up were performed as previously described in Chapter 2. The pre-operative systemic inflammatory response was measured using the mGPS and NPS as previously described in Chapters 2 and 5 respectively.

Electronic patient case notes were reviewed for pre-operative use of aspirin and statins. Primary care referral letters were the primary source of prescribing data. Pre-operative anaesthetic assessment documents and medical clerk-in documents completed on admission to hospital were used if referral letters did not include the appropriate information. For the purposes of the present study, patients who were prescribed aspirin or statins at the time of surgery were considered as aspirin or statin users. Patient comorbidity using ASA Physical Status grade, smoking status and body mass index (BMI) was all obtained from pre-operative anaesthetic assessments.

Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 30th May 2015 that served as the censor date. Cancer-specific survival was measured from the date of surgery

until the date of death from recurrent or metastatic colorectal cancer, and overall survival until date of death from any cause.

Statistical analysis

The relationship between clinicopathological characteristics and aspirin and statin use was examined using the χ^2 method for linear trend for categorical variables. A *P*-value ≤0.05 was considered statistically significant. Binary logistic regression was used to examine the relationship between aspirin and statin use, clinicopathological characteristics and the presence of a systemic inflammatory response, as characterised by mGPS≥1, by calculating ORs and 95% CIs. Variables with *P*- value ≤0.1 on univariate analysis were entered into a multivariate model using a backwards conditional method. The relationship between aspirin use and survival was examined using Kaplan-Meier log-rank analysis. Survival was displayed as percentage surviving to three years (SE). All analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). The West of Scotland Research Ethics Committee approved the study.

10.3 Results

Four hundred and forty-six patients undergoing potentially curative resection of stage I-III colorectal cancer were included. Demographics of the study population are displayed in Table 10.1 and Table 10.2. Approximately two thirds of patients were 65 or older at time of resection and 57% were male. Ten percent of patients underwent emergency resection and 14% underwent neoadjuvant chemoradiotherapy prior to surgery. One third of patients underwent resection of rectal cancer. Pathological staging confirmed stage I disease in 103 (23%) patients, stage II disease in 173 (39%) patients and stage III disease in 160 (36%) patients. Of the 60 patients who received neoadjuvant therapy, 10 had evidence of pathological complete response. A quarter of patients had an elevated mGPS and 16% had an elevated NPS prior to surgery. Twenty-seven percent of patients were prescribed aspirin and 42% were prescribed a statin at the time of diagnosis. All patients were prescribed once daily low-dose aspirin (75mg) and a number of different statin types and doses were prescribed.

The relationship between aspirin and statin use and clinicopathological characteristics

Aspirin and statin use was strongly associated, with 100 patients receiving both medications ($P<0.001$, Table 10.3). The relationship between aspirin and statin use and clinical characteristics is displayed in Table 10.1. Aspirin use was associated with older age at diagnosis ($P=0.001$), male sex ($P<0.05$), higher ASA grade ($P<0.001$), higher BMI ($P<0.05$) and showed a trend towards higher prevalence of ever smoking ($P=0.052$). Furthermore, aspirin use was significantly inversely associated with the presence of a systemic inflammatory response as measured by mGPS (mGPS=0 84% vs. 71%, $P=0.007$) but not the NPS ($P=0.746$). Statin use was associated with older age, higher ASA grade (both $P<0.001$) and elevated BMI ($P<0.01$) and showed a relationship to less frequent use

of neoadjuvant therapy (P<0.01). No relationship between statin use and either mGPS or NPS was observed.

The relationship between aspirin and statin use and pathological characteristics is displayed in Table 10.2. Aspirin use was not associated with pathological characteristics of the tumour. Statin use was inversely associated with the presence of margin involvement (P<0.05) but showed no other association with pathological characteristics.

When analysis was restricted to patients undergoing elective resection without neoadjuvant therapy, aspirin use remained associated with the presence of a systemic inflammatory response as measured by mGPS (P=0.019) but not the NPS. Statin use was not associated with either the mGPS or NPS.

The relationship between aspirin and statin use and the pre-operative systemic inflammatory response in patients undergoing potentially curative resection of colorectal cancer

The relationship between aspirin and statin use, host and tumour characteristics and the presence of a pre-operative systemic inflammatory response was subsequently examined using binary logistic regression (Table 10.4). For the purposes of analysis, the presence of a systemic inflammatory response was defined as mGPS≥1. On univariate analysis, emergency presentation (OR 8.36, P<0.001), advancing T stage (OR 3.13, P<0.001), advancing N stage (OR 1.32, P=0.066), poor differentiation (OR 3.09, P=0.001), margin involvement (OR 4.31, P=0.001), peritoneal involvement (OR 4.37, P<0.001) and tumour perforation (OR 9.35, P=0.007) were associated with the presence of an elevated mGPS, whereas elevated BMI (OR 0.68, P<0.01), neoadjuvant therapy (OR 0.48, P=0.054), rectal primary (OR 0.37, P<0.001) and aspirin use (OR 0.47, P=0.007) were inversely associated with the mGPS. Statin use was not associated with the mGPS on univariate analysis. On multivariate analysis, aspirin use was associated with a lower mGPS (OR 0.38, P=0.005), independent of emergency presentation (OR 4.00, P=0.001), rectal primary (OR 0.58,

P=0.091), T stage (OR 1.9, *P*<0.001), differentiation (OR 2.06, *P*=0.069) and margin involvement (OR 3.28, *P*=0.033).

When analysis was restricted to patients undergoing elective resection without neoadjuvant therapy (Table 10.5), advancing age (*P*=0.066), BMI, T stage, differentiation, margin involvement, peritoneal involvement (all *P*<0.01) and aspirin use (*P*<0.05) were associated with mGPS on univariate analysis. On multivariate binary logistic regression, BMI (OR 0.66, *P*<0.05), T stage (OR 2.45, *P*<0.001) and aspirin use (OR 0.41, *P*<0.05) were independently associated with the presence of a systemic inflammatory response as measured by mGPS.

The relationship between aspirin use and survival of patients undergoing potentially curative resection of colorectal cancer

The relationship between pre-operative aspirin use, systemic inflammatory responses and three-year cancer-specific and overall survival following elective resection of colorectal cancer without prior neoadjuvant therapy was subsequently examined. Three patients who were lost to follow-up and three patients who died within 30 days of surgery were excluded from survival analysis. The median follow-up of survivors was 33 months (interquartile range 21-44 months) with 30 cancer-associated deaths and 14 non-cancer deaths. Aspirin use was associated with poorer three-year cancer-specific survival (93% (2) vs. 85% (4), *P*=0.043) and was associated with poorer three-year overall survival (92% (2) vs. 75% (5), *P*=0.001). When stratified by pre-operative mGPS (Figure 10.1), in patients with mGPS=0, aspirin use was associated with reduced cancer-specific survival (95% (2) vs. 87% (5), *P*=0.032) and overall survival (94% (2) vs. 75% (6), *P*<0.001). In patients with mGPS≥1, aspirin use showed a non-significant trend towards reduced cancer-specific (88% (4) vs. 71% (14), *P*=0.333) and overall survival (83% (5) vs. 71% (14), *P*=0.540).

To control for the interrelationship between aspirin use and patient comorbidity, subsequent analysis was performed on patients with ASA grade I/II. Aspirin use was not associated with cancer-specific survival (95% (2) vs. 91% (6), P=0.481), but was associated with reduced overall survival (94% (2) vs. 83% (7), P=0.05). When stratified by the systemic inflammatory response, in patients with mGPS=0, aspirin use was not associated with cancer-specific survival (96% (2) vs. 90% (7), P=0.160) but was associated with reduced overall survival (96% (2) vs. 80% (8), P=0.004). The number of patients with mGPS≥1 who were prescribed aspirin (n=5) precluded meaningful statistical analysis, however there were no cancer-associated or non-cancer deaths in this group within three years of surgery, compared to three cancer-associated deaths in patients with mGPS≥1who were not prescribed aspirin (n=36).

10.4 Discussion

Although not associated with the pathological characteristics of the tumour, patients prescribed regular low-dose aspirin prior to potentially curative resection of stage I-III colorectal cancer were less likely to have elevated systemic inflammatory responses than those not prescribed aspirin. The results of the present study suggest that the protective effects of aspirin in patients with colorectal cancer may, at least in part, be mediated by attenuation of aberrant, tumour-associated host inflammatory responses.

In the present study, all patients prescribed aspirin received low-dose treatment (75mg) which, similar to statins, is primarily utilised for primary and secondary prevention of cardiovascular disease. Reflective of this, patients prescribed aspirin and statins were more likely to have a higher ASA grade at time of surgery and were more likely to be older. Given that both a high burden of comorbidity and advanced age have previously been associated with the presence of an elevated mGPS in patients with colorectal cancer (392), the present results, whereby aspirin use was associated with a low mGPS even after controlling for these factors, are intriguing. Indeed, given that patients prescribed these drugs would be expected to be systemically inflamed due to underlying co-morbidities, the absolute reduction of 13% in the proportion of patients with an elevated mGPS may under-estimate the true anti-inflammatory effect of aspirin in patients with colorectal cancer.

Of interest however, the ASA grade was not associated with the pre-operative systemic inflammatory response in the present analysis as might be expected. This may reflect the subjective nature of assessment of the ASA grade (527). Indeed, although the ASA grade may be a reliable indicator of in-hospital risk (528, 529), it does not fully evaluate patient co-morbid status (530). As such, further studies considering the burden of co-morbid disease should utilise more rigorous, objective measures, such as the Lee Cardiac Risk Index (392).

Increased adiposity and an elevated BMI have previously been identified as risk factors for elevated systemic inflammatory responses as evidenced by serum CRP (417, 531). In the present analysis however, elevated BMI was not associated with the systemic inflammatory response, with patients with a low BMI more likely to have an elevated CRP and mGPS; 62% of patients in the low BMI group had a mGPS≥1 compared to only 18% of patients in the high BMI group. This may reflect the underlying nature of the systemic inflammatory response in patients with cancer. It is now accepted that elevated systemic inflammatory responses are an integral component of the cancer cachexia syndrome (399), and may not only drive but be driven by weight loss and myopenia (482). Furthermore, the present study may be underpowered to find any significant relationship between an elevated BMI and a clinically significant elevation of serum CRP>10mg/L; indeed, Visser and colleagues, utilising data from the Third National Health and Nutrition Examination Survey, required a cohort of over 16 000 patients to identify an approximately two-fold increase in the prevalence of a CRP>10mg/L in obese compared to non-obese individuals (417).

In the present analysis, aspirin use was not associated with pathological characteristics of the tumour, such as TNM stage and the presence of venous invasion. This is contradictory to previous studies utilising cancer registry data and community prescription registration data, where the use of aspirin in the year prior to diagnosis was associated with smaller primary tumour size and reduced likelihood of metastatic disease in patients with colorectal and lung cancer (532, 533). However, these previous studies included patients with metastatic disease whereas the present analysis included only patients with stage I-III colorectal cancer. Furthermore, the methods of statistical analysis employed differ; the present study examined for an overall trend between aspirin use and T stage, N stage and TNM stage, whereas Jonsson et al and Pawitan et al utilised multinomial logistic regression to calculate the risk of a higher tumour stage compared to the lowest possible

stage. Utilising this method in the present cohort, the odds ratio of a T4 versus a T1 tumour comparing aspirin users to non-users is 0.51 (95% CI 0.24–1.11, *P*=0.088), similar in magnitude to the studies by Jonsson and Pawitan (OR 0.66 and OR 0.70 respectively).

The mechanisms responsible for the anti-neoplastic effects of aspirin in patients with cancer and colorectal cancer in particular remain to be fully defined, however the results of the present analysis further support the hypothesis that these may be in part mediated by the host inflammatory response. Aspirin may affect tumour biology and subsequent host inflammatory responses through inhibition of the pro-inflammatory COX-2 enzyme; alongside reducing the incidence of COX-2 expressing tumours (315), previous studies have suggested a survival benefit for aspirin commenced following a diagnosis of colorectal cancer only in patients with COX-2 expressing tumours (296).

Despite evidence supporting a role for COX-2 inhibition, aspirin used at the low doses documented in the present study and many of the previous studies confirming a survival benefit does not irreversibly inhibit tissue COX-2 activity due to its short half-life, and as such other putative mechanisms have been suggested (534). One potential candidate downstream of COX-2 activity is inactivation of the P13K pathway, which is constitutively activated in patients with mutated *PIK3CA*. Analysis of data from the Nurses' Health Study and Health Professionals Follow-up Study (208), and post-hoc molecular analysis of data from the VICTOR randomised controlled trial (526) both support the presence of *PIK3CA* mutation as a predictive biomarker for adjuvant aspirin therapy. Furthermore, both pre-clinical data (535, 536) and analysis of STAT3-associated SNPs in patients with breast cancer (537) have suggested a potential role of the JAK/STAT3 pathway in mediating the anti-tumour effects of aspirin.

In addition to a direct effect on the tumour cell, irreversible inhibition of platelet function may also mediate the anti-tumour effects of low dose aspirin. Platelet function is enhanced

in patients with colorectal cancer (319); furthermore, platelets aggregate with circulating tumour cells to facilitate dissemination of micrometastases (538), and may mediate the process of EMT through activation of the NF-κB pathway (525). Indeed, Reimers and colleagues hypothesised that such activity may explain the relationship between aspirin and increased survival in patients with tumours which express HLA-1, a necessary prerequisite for tumour cell-platelet signalling (525). Similarly, platelet activation may also activate STAT3 through IL-6 transignalling, which again is attenuated by inhibition of platelet function (539, 540). Indeed, given that the above putative mechanisms primarily rely on inactivation of signal transduction pathway, the presently observed associations with acute phase proteins but not cellular components of the systemic inflammatory response, such as platelet count, is not surprising.

The present analysis found no consistent relationship between statin use and either tumour pathological characteristics or components of the systemic inflammatory response. The evidence supporting the chemotherapeutic effects of statins is more conflicting. Whereas a previous non-randomised controlled study found that pre-operative simvastatin use decreased serum IL-6 concentrations in patients with colorectal cancer (358), a further cohort study of patients undergoing chemoradiotherapy for oesophageal and rectal cancer found no such relationship (359). The results of such cohort-based studies, however, may be confounded by other variables which have not been controlled for, such as prescribing of other medications with potential chemotherapeutic benefit (518, 541). In the present analysis, for example, statin and aspirin use were strongly associated. Furthermore, the type of statin used may also influence chemotherapeutic benefit; whereas in the present study all statin types were considered, previous studies have suggested that only lipophilic statins, which may easily cross the cell membrane, are associated with increased survival in patients with colorectal cancer (524, 542).

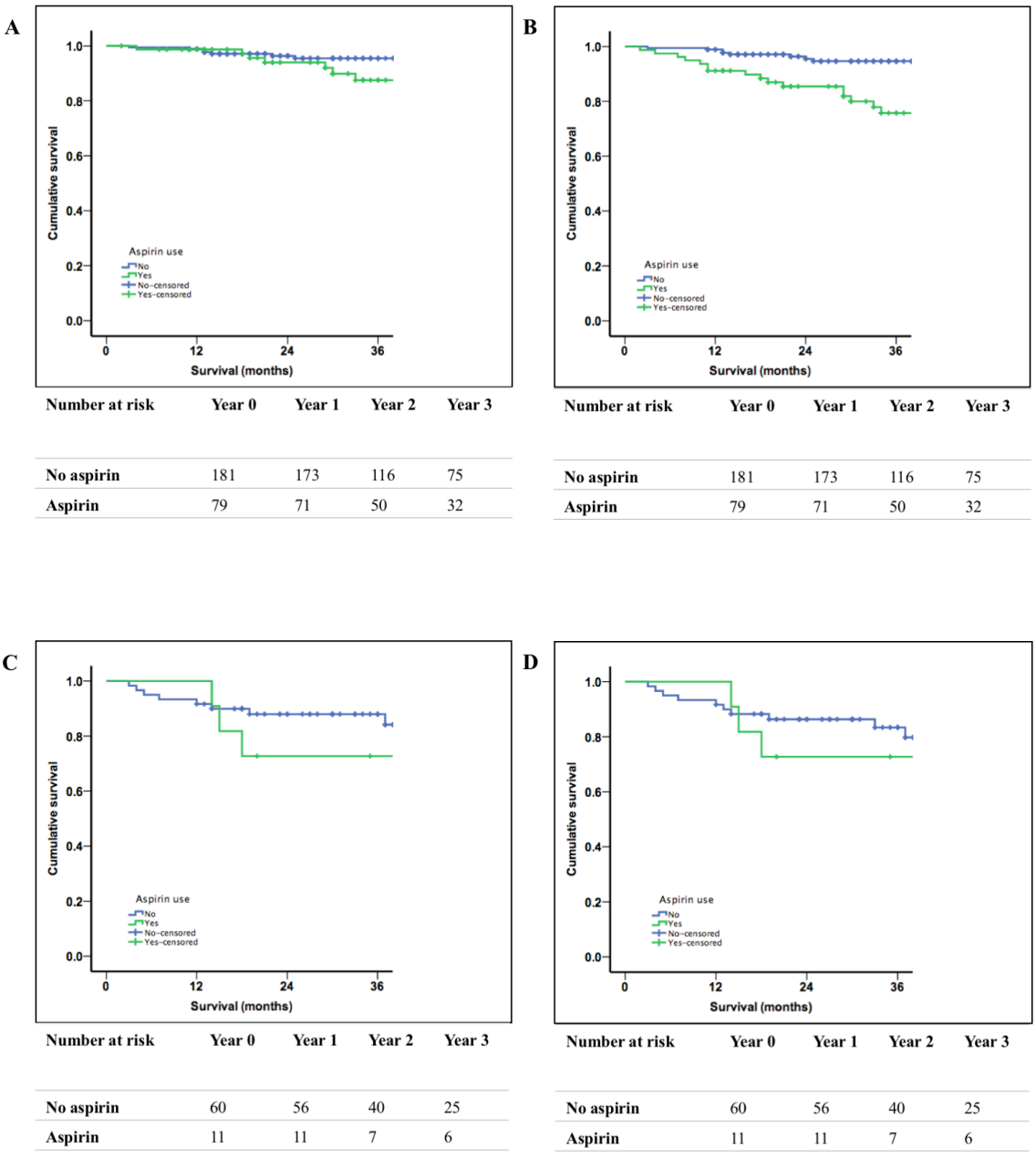
Despite being associated with a lower mGPS, aspirin use was not associated with improved survival and in fact appeared to be associated with poorer cancer-specific and overall survival at three years. These results must be interpreted with caution due to the lack of long-term follow-up, the limited number of observed events, and the small number of patients with an elevated mGPS and pre-diagnosis aspirin use, however this may reflect the underlying indication for receiving aspirin; most if not all patients will have received aspirin for secondary prevention of cardiovascular disease rather than for its chemotherapeutic effects. As such any effect on survival will be confounded by the presence of co-morbidities.

Although aspirin use is associated with reduced incidence of colorectal cancer, an increasing body of evidence suggests that it is potentially aspirin use commenced after, and not prior to diagnosis, which is associated with improved survival of patients with colorectal cancer (521). Indeed, although the results of the present analysis suggest that pre-diagnosis aspirin use may attenuate or prevent elevated host systemic inflammatory responses, it is not known whether this translates into a long-term improvement in survival. Furthermore, whether the presence of an elevated systemic inflammatory response may be a reliable biomarker of response to adjuvant aspirin therapy remains to be seen, and may be answered by subgroup analysis of the presently recruiting ASCOLT and Add-Aspirin Trials (303, 543).

The present study is limited by the methodology employed to identify aspirin and statin users. Both duration of use and compliance with prescribed medications were not taken into consideration and may confound results. However, the abolishment of prescription charges in Scotland in 2011 means that prescription cost is unlikely to be a barrier to long-term medication. Furthermore, such factors would likely result in an under-estimation of the effects of aspirin and statins on host inflammatory responses. An additional limitation of the present study is that over the counter use of aspirin and other NSAIDs was not taken

into consideration. However, previous data has shown that over 90% of aspirin use is from prescribed use (544), and reporting of prescribed NSAID use in the present cohort was low (<8%), suggesting that this is unlikely to be a confounding factor.

In conclusion, the present results suggest that the anti-neoplastic effects of aspirin may be mediated in part through attenuation of host inflammatory responses. These results provide a rationale for future clinical trials examining the effects of aspirin therapy in patients with colorectal cancer and elevated systemic inflammatory responses.



Number at risk	Year 0	Year 1	Year 2	Year 3
No aspirin	181	173	116	75
Aspirin	79	71	50	32

Number at risk	Year 0	Year 1	Year 2	Year 3
No aspirin	181	173	116	75
Aspirin	79	71	50	32

Number at risk	Year 0	Year 1	Year 2	Year 3
No aspirin	60	56	40	25
Aspirin	11	11	7	6

Number at risk	Year 0	Year 1	Year 2	Year 3
No aspirin	60	56	40	25
Aspirin	11	11	7	6

Figure 10.1 The relationship between aspirin use, modified Glasgow Prognostic Score and survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** mGPS=0 and cancer-specific survival (*P*=0.032), **(B)** mGPS=0 and overall survival (*P*<0.001), **(C)** mGPS≥1 and cancer-specific survival (*P*=0.333), and **(D)** mGPS≥1 and overall survival (*P*=0.540). All *P*-values calculated using log-rank analysis

Table 10.1 The relationship between aspirin and statin use and clinical characteristics of patients undergoing potentially curative resection of stage I-III colorectal cancer

		All	Aspirin use			Statin use		
Clinical characteristics (*n* when data missing)		**(*n*=446) (%)**	**No aspirin (*n*=326) (%)**	**Aspirin (*n*=120) (%)**	***P***	**No statin (*n*=259) (%)**	**Statin (*n*=187) (%)**	***P***
Age					0.001			<0.001
	<65	158 (35)	135 (41)	23 (19)		114 (44)	44 (24)	
	65-74	170 (38)	110 (34)	60 (50)		92 (35)	78 (41)	
	>74	118 (27)	81 (25)	37 (31)		53 (21)	65 (35)	
Sex					0.014			0.117
	Female	191 (43)	151 (46)	40 (33)		119 (46)	72 (39)	
	Male	255 (57)	175 (54)	80 (67)		140 (54)	115 (61)	
ASA grade					<0.001			<0.001
(436)	**I-II**	280 (64)	234 (73)	46 (40)		195 (77)	85 (47)	
	III-IV	156 (36)	86 (27)	70 (60)		59 (23)	97 (53)	
BMI					0.049			0.004
(415)	**<18.5**	13 (3)	12 (4)	1 (1)		9 (4)	4 (2)	
	18.5-24.9	131 (32)	102 (33)	29 (26)		88 (37)	43 (24)	
	25-29.9	128 (31)	91 (30)	37 (34)		69 (29)	59 (33)	
	>30	143 (34)	100 (33)	43 (39)		72 (30)	71 (40)	
Smoking status					0.052			0.272
(436)	**Never**	208 (48)	164 (51)	44 (37)		132 (52)	76 (42)	
	Ex	167 (38)	110 (35)	57 (48)		85 (33)	82 (45)	
	Current	61 (14)	44 (14)	17 (14)		38 (15)	23 (13)	
Presentation					0.511			0.885
	Elective	402 (90)	292 (90)	110 (92)		233 (90)	169 (90)	
	Emergency	44 (10)	34 (10)	10 (8)		26 (10)	18 (10)	
Neoadjuvant therapy					0.562			0.004
	No	386 (86)	284 (87)	102 (85)		214 (83)	172 (92)	
	Yes	60 (14)	42 (13)	18 (15)		45 (17)	15 (8)	

Table 10.1 (continued) The relationship between aspirin and statin use and clinical characteristics of patients undergoing potentially curative resection of stage I-III colorectal cancer

		All	Aspirin use			Statin use		
Clinical characteristics (*n* when data missing)		**(*n*=446) (%)**	**No aspirin (*n*=326) (%)**	**Aspirin (*n*=120) (%)**	***P***	**No statin (*n*=259) (%)**	**Statin (*n*=187) (%)**	***P***
mGPS					0.007			0.415
(435)	**0**	326 (75)	226 (71)	100 (84)		184 (73)	142 (77)	
	1	40 (9)	32 (10)	8 (7)		25 (10)	15 (8)	
	2	69 (16)	58 (18)	11 (9)		42 (17)	27 (15)	
NPS					0.746			0.619
(438)	**0**	366 (84)	265 (83)	101 (86)		213 (84)	153 (83)	
	1	57 (13)	45 (14)	12 (10)		34 (13)	23 (13)	
	2	15 (3)	10 (3)	5 (4)		7 (3)	8 (4)	

Data analysed using χ^2 analysis for linear trend.

Table 10.2 The relationship between aspirin and statin use and tumour characteristics of patients undergoing potentially curative resection of stage I-III colorectal cancer

		All	Aspirin use			Statin use		
Tumour characteristics (*n* when data missing)		**(*n*=446) (%)**	**No aspirin (*n*=326) (%)**	**Aspirin (*n*=120) (%)**	***P***	**No statin (*n*=259) (%)**	**Statin (*n*=187) (%)**	***P***
Tumour site					0.364			0.111
	Colon	301 (67)	224 (69)	77 (64)		167 (64)	134 (72)	
	Rectum	145 (33)	102 (31)	43 (36)		92 (36)	43 (28)	
Stage					0.901			0.862
	PCR	10 (2)	8 (3)	2 (2)		8 (3)	2 (1)	
	I	103 (23)	74 (23)	29 (24)		57 (22)	46 (25)	
	II	173 (39)	126 (39)	47 (39)		101 (39)	72 (38)	
	III	160 (36)	118 (36)	42 (35)		93 (36)	67 (36)	
T stage					0.459			0.932
	0	10 (2)	8 (3)	2 (2)		8 (3)	2 (1)	
	1	60 (13)	41 (13)	19 (16)		32 (12)	28 (15)	
	2	61 (14)	48 (15)	13 (11)		39 (15)	22 (12)	
	3	223 (50)	155 (47)	68 (56)		123 (48)	100 (53)	
	4	92 (21)	74 (23)	18 (15)		57 (22)	35 (18)	
N stage					0.916			0.858
	0	286 (64)	208 (64)	78 (65)		166 (64)	120 (64)	
	1	107 (24)	81 (25)	26 (22)		61 (24)	46 (25)	
	2	53 (12)	37 (11)	16 (13)		32 (12)	21 (11)	
Less than 12 nodes					0.478			0.127
examined	**No**	362 (81)	262 (80)	100 (83)		204 (79)	158 (84)	
	Yes	84 (19)	64 (20)	20 (17)		55 (21)	29 (16)	
Differentiation					0.616			0.795
(432)	**Mod-well**	390 (90)	283 (90)	107 (91)		224 (90)	166 (91)	
	Poor	42 (10)	32 (10)	10 (9)		25 (10)	17 (9)	

Table 10.2 (continued) The relationship between aspirin and statin use and pathological characteristics of patients undergoing potentially curative resection of stage I-III colorectal cancer

		All	Aspirin use			Statin use		
Tumour characteristics (*n* when data missing)		**(*n*=446) (%)**	**No aspirin (*n*=326) (%)**	**Aspirin (*n*=120) (%)**	***P***	**No statin (*n*=259) (%)**	**Statin (*n*=187) (%)**	***P***
Venous invasion					0.164			0.127
	Absent	177 (40)	123 (308)	54 (45)		95 (37)	82 (44)	
	Present	269 (60)	203 (62)	66 (55)		164 (63)	105 (56)	
Margin involvement					0.743			0.030
	Absent	425 (95)	310 (95)	115 (96)		242 (93)	183 (98)	
	Present	21 (5)	16 (5)	5 (4)		17 (7)	4 (2)	
Peritoneal involvement					0.109			0.811
	Absent	365 (92)	261 (80)	104 (87)		211 (81)	154 (82)	
	Present	81 (18)	65 (20)	16 (13)		48 (19)	33 (18)	
Tumour perforation					0.496			0.641
	Absent	438 (98)	321 (98)	117 (97)		255 (98)	183 (98)	
	Present	8 (2)	5 (2)	3 (3)		4 (2)	4 (2)	

Data analysed using χ^2 analysis for linear trend.

Table 10.3 The relationship between aspirin and statin use in patients undergoing potentially curative resection of stage I-III colorectal cancer

	Aspirin ***n*** **(%)**	**Statin** ***n*** **(%)**
All patients	120	187
Aspirin		
No	-	87 (47)
Yes	-	100 (53)
Statin		
No	20 (17)	-
Yes	100 (83)	-

Table 10.4 Determinants of the pre-operative modified Glasgow Prognostic Score of patients undergoing potentially curative resection of stage I-III colorectal cancer

	mGPS=0	mGPS≥1	Univariate OR (95% CI)	*P*	Multivariate OR (95% CI)	*P*
Age (<65/ 65-74/ >74)	114 / 132 / 80	39 / 33/ 38	1.17 (0.89-1.54)	0.272	-	-
Sex (female/ male)	141 / 185	47 / 63	1.02 (0.66-1.58)	0.924	-	-
ASA grade (I-II/ III-IV)	207 / 68	117 / 38	0.99 (0.63-1.56)	0.961	-	-
BMI (<18.5/18.5-24.9/25-29.9/>30)	5 / 94 / 99 / 113	8 / 35 / 26 / 25	0.68 (0.52-0.88)	0.004	-	0.190
Smoking status (never/ ever)	157 / 164	45 / 60	1.28 (0.82-1.99)	0.281	-	-
Presentation (elective/ emergency)	312 / 14	80 / 30	8.36 (4.23-16.50)	<0.001	4.00 (1.74-9.20)	0.001
Neoadjuvant therapy (no/ yes)	275 / 51	101 / 9	0.48 (0.23-1.01)	0.054	-	0.821
Tumour site (colon/ rectum)	203 / 123	90 / 20	0.37 (0.22-0.63)	<0.001	0.58 (0.31-1.09)	0.091
T stage (0/ 1/ 2/ 3/ 4)	10 / 51 / 57 / 166 / 42	0 / 5 / 2 / 54 / 49	3.13 (2.23-4.40)	<0.001	1.93 (1.36-2.75)	<0.001
N stage (0/ 1/ 2)	212 / 82 / 32	66 / 23 / 21	1.32 (0.98-1.77)	0.066	-	0.131
Less than 12 nodes (no/ yes)	263 / 63	92 / 18	0.82 (0.46-1.45)	0.490	-	-
Differentiation (mod-well/ poor)	292 / 21	90 / 20	3.09 (1.60-5.96)	0.001	2.06 (0.95-4.50)	0.069
Venous invasion (absent/ present)	135 / 191	38 / 72	1.34 (0.85-2.10)	0.204	-	-
Margin involvement (absent/ present)	317 / 9	98 / 12	4.31 (1.77-10.54)	0.001	3.28 (1.10-9.76)	0.033
Peritoneal involvement (absent/ present)	287 / 39	69 / 41	4.37 (2.62-7.29)	<0.001	-	0.130
Tumour perforation (absent/ present)	324 / 2	104 / 6	9.35 (1.86-47.01)	0.007	-	0.238
Aspirin (no/ yes)	226 / 100	91 / 19	0.47 (0.27-0.82)	0.007	0.38 (0.20-0.75)	0.005
Statin (no/ yes)	184 / 142	68 / 42	0.80 (0.51-1.25)	0.324	-	-

Data analysed using binary logistic regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

Table 10.5 Determinants of the pre-operative modified Glasgow Prognostic Score of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

	Univariate OR (95% CI)	*P*	Multivariate OR (95% CI)	*P*
Age (<65/ 65-74/ >74)	1.38 (0.99-1.94)	0.066	-	0.436
Sex (female/ male)	1.19 (0.70-2.03)	0.522	-	-
ASA grade (I-II/ III-IV)	1.20 (0.86-1.67)	0.295	-	-
BMI (<18.5/ 18.5-24.9/ 25-29.9/ >30)	0.60 (0.44-0.82)	0.002	0.66 (0.47-0.92)	0.013
Smoking status (never/ ever)	1.56 (0.91-2.66)	0.103	-	-
Tumour site (colon/ rectum)	0.60 (0.31-1.16)	0.129	-	-
T stage (1/ 2/ 3/ 4)	2.87 (1.93-4.28)	<0.001	2.45 (1.62-3.71)	<0.001
N stage (0/ 1/ 2)	1.11 (0.76-1.63)	0.590	-	-
Less than 12 nodes (no/ yes)	0.74 (0.36-1.50)	0.400	-	-
Differentiation (mod-well/ poor)	3.57 (1.61-7.92)	0.002	-	0.109
Venous invasion (absent/ present)	0.98 (0.57-1.66)	0.925	-	-
Margin involvement (absent/ present)	7.94 (1.93-32.59)	0.004	-	0.188
Peritoneal involvement (absent/ present)	3.21 (1.72-6.00)	<0.001	-	0.230
Tumour perforation (absent/ present)	-	0.999	-	-
Aspirin (no/ yes)	0.42 (0.21-0.83)	0.013	0.41 (0.19-0.89)	0.025
Statin (no/ yes)	0.68 (0.40-1.16)	0.156	-	-

Data analysed using binary logistic regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

11 Pre-operative, colonoscopic-based assessment of the tumour microenvironment in patients with colorectal cancer

11.1 Introduction

In addition to guiding the prognosis of patients with colorectal cancer, assessment of the tumour microenvironment may also be of value in predicting response to treatment. The presence of a high density lymphocytic infiltrate, for example, has been shown to predict increased response to neoadjuvant chemoradiotherapy (512, 545-547). Similarly, treatment effect of anti-angiogenic and other biological agents may also be influenced by the composition of the tumour microenvironment (490, 548).

Furthermore, the tumour microenvironment itself is an attractive therapeutic target, with increasing evidence suggesting that radiotherapy, anti-angiogenic therapies and other novel agents may influence its composition and architecture (490, 549, 550). Furthermore, anti-inflammatory agents, such as NSAIDs (326) and H2RAs (366) have been shown to promote infiltration of the tumour microenvironment by activated lymphocytes.

It is clear that pre-operative assessment of the tumour microenvironment could inform decision-making regarding neoadjuvant therapy prior to surgical resection. For example, identifying patients with an unfavourable tumour microenvironment (i.e. low density inflammatory cell infiltrate and expanded tumour-associated stroma) could potentially avoid the administration of standard chemoradiotherapy regimes where it is unlikely to be of benefit, and instead allow for administration of therapies targeting the tumour microenvironment itself.

One of the inherent difficulties with characterisation of the tumour microenvironment is the reliance on tumour specimens obtained following surgical resection, therefore limiting clinical utility in the neoadjuvant setting. Accordingly, several groups have attempted to characterise the tumour microenvironment of patients with colorectal cancer utilising pre-

operative colonoscopic biopsies, primarily with the aim of predicting response to neoadjuvant chemoradiotherapy (512, 545-547). However, interpretation and subsequent reporting of biopsy specimens depends on a number of factors, including quality and quantity of tissue obtained, tissue fixation and availability of surplus blocks and slides for additional testing (551, 552). Furthermore, given that radiotherapy induces significant histologic reactions and architectural restructuring, it is not clear from these observational studies how closely biopsy-based assessment truly reflects the tumour microenvironment. Two studies included patients proceeding directly to surgery for rectal cancer; whereas one study (n=31) found that biopsy assessment of the inflammatory cell infiltrate correlated with full section analysis (546), another study (n=54) found that the density of $CD8^+$ T-lymphocytes in full sections was significantly higher than that in preoperative biopsies, suggesting that intratumoural heterogeneity may preclude biopsy-based assessment (547). Additionally, although biopsy-based assessment of the tumour-associated stroma in patients with oesophageal cancer has previously been shown to be feasible (553), no study to date has assessed the use of colonoscopic biopsies as a means of pre-operatively assessing TSP in patients with colorectal cancer.

Given the above, the aim of the present study was to examine the feasibility and prognostic utility of pre-operative colonoscopic biopsy-based assessment of the inflammatory cell infiltrate and tumour-associated stroma in patients undergoing potentially curative resection of colorectal cancer without neoadjuvant therapy.

11.2 Patients and Methods

Clinicopathological characteristics

Patients were identified from a prospectively collected database of elective and emergency colorectal cancer resections performed since January 1997 in a single surgical unit in Glasgow Royal Infirmary. Patients who had undergone assessment of the tumour microenvironment as described in Chapter 4, and for whom corresponding pre-operative colonoscopic biopsies were available, were included. Only patients with documented invasive adenocarcinoma present on pre-operative biopsies were included, whereas those with dysplastic changes only were excluded. Indications for adjuvant chemotherapy, MDT review and routine follow-up of patients following surgery has previously been described in Chapter 2. Date and cause of death was crosschecked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 15th March 2013 that served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer.

Biopsy specimens

Archived, paraffin-embedded, colonoscopic biopsy specimens and corresponding H&E-stained sections matched to the primary tumour were retrieved for each patient. Due to the limited quantity of tissue available, only $CD3^+$ T-lymphocytes were assessed in biopsy specimens. Sections (2.5μm thick) were cut and mounted on silanised slides before being dewaxed in xylene and rehydrated through graded alcohols. An autostainer (ThermoFisher Autostainer 480s) was used to perform staining. Antigen retrieval was carried out in a PT module (ThermoFisher) using ThermoFisher retrieve solution pH9. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 5 minutes before rinsing with TBS. Primary antibody ($CD3^+$; ThermoFisher RM-9107-S) was applied (1:300 dilution) and incubated for 20 minutes at room temperature before rinsing again with TBS. The signal was amplified and visualised using the ThermoFisher Quanto kit

and diaminobenzidine colour developer before counterstaining with haematoxylin for three minutes. Sections were then washed in running tap water for one minute and dipped in acid-alcohol for five seconds before washing again. Finally, sections were dipped in Scott's tap water for one minute before rinsing again and dehydrating using graded alcohols and xylene. Cover slips were applied with DPX.

Both H&E and $CD3^+$-stained colonoscopic biopsies and surgically resected specimens were converted to digital format using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, Milton Keynes, UK) at x20 optical magnification. Subsequent visualisation and automated image analysis was performed using Slidepath Digital Image Hub, version 4.0.1 (Slidepath, Leica Biosystems, Milton Keynes, UK).

Manual assessment of the tumour microenvironment

Full section assessment

Full H&E-stained sections of the deepest point of invasion obtained following surgical resection were used to examine the local inflammatory cell infiltrate and tumour-associated stroma. The TSP was assessed as described in Chapter 4 and categorised as low (≤50%) or high (>50%). Sections stained for $CD3^+$ mature T-lymphocytes were used for assessment of the inflammatory infiltrate as described in Chapter 4, and categorised as high and low.

Biopsy assessment

The density of $CD3^+$ T-lymphocytes in colonoscopic biopsies was examined using manual, semi-quantitative assessment. At x10 magnification, the intratumoural density of $CD3^+$ T-lymphocytes throughout the biopsy specimen was graded as low (absent or weak) or high (moderate or strong). T-lymphocytes present within dysplastic and normal mucosa were not considered to be intratumoural lymphocytes and therefore excluded from analysis. An example of colonoscopic biopsy staining is shown in Figure 11.1.

Assessment of the tumour-associated stroma using H&E-stained colonoscopic biopsies was performed using TSP. At x10 magnification, the proportion of intratumoural stroma was graded as low (≤50%) or high (>50%). Where possible, assessment was performed in areas of the biopsy specimen where tumour cells were present circumferentially around the tumour-associated stroma, with mucinous deposits, necrosis and dysplastic or normal mucosa all excluded from the area of analysis. Similar to the technique previously described by Mesker and colleagues for assessment of oesophageal biopsies, stromal fragments without any cancer cells were excluded from analysis (553).

All assessments were performed by an investigator blinded to clinicopathological and outcome data. To ensure consistency of scoring, a proportion of biopsies were double-scored for biopsy T-lymphocyte density (n=28) and TSP (n=30) by a blinded co-investigator (CSDR). The intra-class correlation co-efficient (ICC) was calculated, to assess inter-observer variability, with an ICC 0.40-0.75 considered 'fair to good' (554).

Automated assessment of the tumour inflammatory cell infiltrate

Automated assessment of intratumoural $CD3^+$ T-lymphocyte density using both full sections and colonoscopic biopsies was performed using the Tissue Image Analysis, version 2.0 plugin for Slidepath (Slidepath, Leica Biosystems, Milton Keynes, UK). To ensure only intratumoural T-lymphocytes were analysed in surgically resected specimens, sections were annotated; at x10 magnification, three $1mm^2$ rectangles were drawn using the *'Draw rectangle'* tool to include regions felt to be most representative of the tumour microenvironment. Using these annotated regions, *'Tissue IA Optimiser'* was selected and *'Measure stained cells algorithm'* was chosen. The algorithm provides a range of values to describe the number of positive cells in the region of interest. For the purposes of the present study, the *"cellular H-score of nuclear staining"* (H-score) was recorded for each annotated area and the average H-score calculated for each specimen. A similar method

was used to calculate T-lymphocyte density of colonoscopic biopsies. Due to the area of biopsy material available for analysis for each specimen however, only one $1mm^2$ rectangle was drawn per section. An example of automated assessment of colonoscopic biopsy T-lymphocyte density is shown in Figure 11.1.

Statistical analysis

Receiver operating characteristic (ROC) curves and the area under the curve (AUC) was used to identify the optimal cut-off point for the H-score as a predictor of high T-lymphocyte density within the tumour microenvironment of surgically resected specimens. The relationship between categorical variables was examined using the χ^2 method for linear trend and continuous variables using Mann-Whitney U test. Cancer-specific survival was displayed as percentage surviving at five years (SE). The relationship between clinicopathological characteristics and cancer-specific survival was examined using Kaplan-Meier log-rank analysis and multivariate Cox proportional hazards regression to calculate HRs and 95% CIs. Variables with a *P*-value ≤ 0.1 on univariate regression analysis were entered into a multivariate model using a backward conditional method. All statistical analyses were performed using SPSS version 22.0 for Mac (IBM SPSS, IL, USA). A *P*-value ≤ 0.05 was considered statistically significant.

11.3 Results

One hundred and twenty patients were included in the analysis. Clinicopathological characteristics are displayed in Table 11.1. Almost two thirds of patients were 65 years of age or older at time of surgery and 53% were male. Eighty-two (68%) patients had a colon cancer. Six patients (5%) had stage I disease, whereas 55 (46%) and 59 (49%) had stage II and stage III disease respectively. Thirty-seven (31%) patients received adjuvant therapy. Using manual assessment of surgically resected full sections, a high T-lymphocyte density within the invasive margin, tumour stroma and cancer cell nests was identified in 45%, 55% and 33% patients of patients respectively. A high TSP was identified in 27 (23%) patients.

Biopsy-based, manual assessment of the tumour microenvironment

Semi-quantitative, manual assessment of colonoscopic biopsy T-lymphocyte density was performed. The interobserver ICC was 0.585 (P=0.014) indicating fair-to-good concordance. Fifty-five patients (46%) had a high density of T-lymphocytes. Biopsy T-lymphocyte density was strongly associated with T-lymphocyte density at the invasive margin (P=0.006) and within the cancer cell nests (P=0.046) of full section specimens, but not within the cancer stroma (P=0.313; Table 11.2).

Assessment of TSP using colonoscopic biopsies was performed. The interobserver ICC was 0.745 (P<0.001), indicating good concordance. Sixty-five patients (54%) had a high TSP. Biopsy TSP was associated with full section TSP (P=0.001; Table 11.2).

Biopsy-based, automated assessment of the tumour microenvironment

To confirm that automated assessment of T-lymphocyte density correlated with manual assessment, automated assessment was first performed on surgically resected, full section specimens. The median H-score was 22 (range 2-94, interquartile range (IQR) 13-38).

The H-score of patients with a high T-lymphocyte density at the invasive margin, within the cancer stroma and the cancer cell nests was significantly higher than those with a low T-lymphocyte density (all $P<0.001$, Table 11.3); the AUC for full section H-score predicting a high T-lymphocyte density was >0.78 ($P<0.001$) for each location within the tumour microenvironment (Figure 11.2, Table 11.3). This analysis confirmed that automated assessment of T-lymphocyte density within surgically resected specimens using the Tissue Image Analysis plugin was comparable to manual, semi-quantitative assessment as previously described.

Automated assessment of colonoscopic biopsy T-lymphocyte density was subsequently performed. Although generally higher, colonoscopic biopsy H-score correlated with full section H-score ($r=0.329$, $P<0.001$). The median H-score was 58 (range 1-140, IQR 33-83). The biopsy H-score of patients with a high density of T-lymphocytes at the invasive margin, within the cancer stroma and the cancer cell nests as assessed manually using full sections was significantly higher than those with a low T-lymphocyte density (all $P<0.05$, Table 11.4). The AUC for biopsy H-score predicting a high T-lymphocyte density was 0.651, 0.677 and 0.622 for the invasive margin, cancer stroma and cancer cell nests respectively, with an optimal cut-off point for each of 57 (Figure 11.3, Table 11.4).

Give the above, the median biopsy H-score was subsequently used to stratify patients into those with a high biopsy T-lymphocyte density (H-score>57) and those with a low biopsy T-lymphocyte density (h-score≤57). Using this threshold (Table 11.5), biopsy H-score was associated with a high density of T-lymphocytes within surgically resected specimens at the invasive margin ($P<0.01$) and cancer stroma ($P<0.001$) and showed a trend towards an association with the density within cancer cell nests ($P=0.060$).

The relationship between colonoscopic biopsy-based assessment of the tumour microenvironment and survival

The relationship between colonoscopic biopsy-based assessment of the tumour microenvironment and cancer-specific survival was examined (Figure 11.4). The median follow-up of survivors was 136 months (interquartile range 115-161 months) with 34 cancer-associated deaths. Manual assessment of colonoscopic biopsy T-lymphocyte density showed a trend towards an association with cancer-specific survival (P=0.120); five-year survival of patients with a high density was 85% (5) compared to 72% (6) for patients with a low density. Automated assessment of biopsy T-lymphocyte density was associated with survival (P=0.007); five-year survival of patients with a high density was 88% (4) compared to 68% (6) for patients with a low T-lymphocyte density. Assessment of biopsy TSP was associated with cancer-specific survival (P=0.005); five-year survival of patients with a low TSP was 84% (4) compared to 68% (7) for patient with a high TSP.

On univariate Cox regression survival analysis (Table 11.6), manual assessment of biopsy T-lymphocyte density showed a trend towards an association with cancer-specific survival (HR 0.58, 95%CI 0.291.17, P=0.125). Automated assessment of biopsy T-lymphocyte density was significantly associated with survival (HR 0.39, 95%CI 019-0.80, P=0.010), as was assessment of biopsy TSP (HR 2.56, 95%CI 1.30-5.04, P=0.007). On multivariate survival analysis, automated biopsy T-lymphocyte density (P<0.05) and biopsy TSP (P<0.01) were associated with cancer-specific survival independent of TNM stage, venous invasion (both P<0.05) and margin involvement (P=0.058).

As automated assessment of biopsy T-lymphocyte density and biopsy TSP were independently associated with survival, the prognostic value of a biopsy-derived Glasgow Microenvironment Score (biopsy GMS) was examined (Figure 11.5). The biopsy GMS was derived as follows: patients with a high automated T-lymphocyte density were given a score of 0, patients with a low automated T-lymphocyte density and low TSP were given a

score of 1, and patients with a low automated T-lymphocyte density and high TSP were given a score of 2. The biopsy GMS stratified cancer-specific survival of patients (P<0.001); patients with biopsy GMS=0 (n=62) had five-year survival of 88% (4), whereas patients with a biopsy GMS=1 (n=38) and 2 (n=20) had five-year survival of 76% (7) and 49% (12) respectively. In this cohort, this was similar to the conventional GMS (GMS=0 89% (5), GMS=1 79% (6), GMS=2 50% (13), P=0.029) and a derived GMS using T-lymphocyte density at the invasive margin and TSP (89% (4), 74% (6) and 49% (14), *P*=0.019).

11.4 Discussion

The results of the present study suggest that pre-operative assessment of the tumour microenvironment is feasible utilising colonoscopic biopsies. Furthermore, such biopsy-based assessments appear to have prognostic value in addition to standard, TNM-based clinicopathological staging. Introduction of pre-operative assessment of the tumour microenvironment may have significant impact not only on staging of patients with colorectal cancer, but also the provision of neoadjuvant therapy, particularly aimed at targeting the tumour microenvironment.

Manual, semi-quantitative assessment of biopsy T-lymphocyte density and TSP predicted both T-lymphocyte density and TSP within surgically resected specimens. The techniques employed were adapted from those previously described for assessment of full sections of tumours incorporating the invasive margin at the deepest point of invasion. Given that biopsy specimens are obtained from the luminal margin of the tumour rather than the invasive margin, this further supports previous work suggesting that lymphocyte density is consistent across different regions of the tumour microenvironment (433). Indeed, this would further advocate the use of biopsy-derived specimens for assessment of the tumour microenvironment when full surgically resected specimens are not available, such as in patients with overt metastatic disease at presentation.

In keeping with previous work in oesophageal cancer (553), there was excellent inter-observer agreement with respect to biopsy TSP. However, manual assessment of biopsy T-lymphocyte density appeared to show greater inter-operator variability, albeit still with 'fair-to-good' agreement as measured by ICC. This may reflect the inherent difficulties of assessment of colonoscopic biopsies which vary not only in size and quality of tissue, but also abundance of tumour tissue present (551). Whereas the only criteria for biopsies to be included in the present analysis was the presence of invasive malignancy, more rigorous

criteria may result in more accurate assessment with improved inter-observer agreement. For example, Courrech Staal and colleagues reported that stringent selection criteria for biopsy sections (at least 20% of invasive malignancy present in the biopsy and at least six fragments present) increased concordance with full section analysis for mutational analysis (553). Furthermore, standardisation of scoring techniques to ensure agreement between investigators should be addressed to ensure consistency of scoring prior to further study.

Digital pathology has been advocated as an important development in the field of routine gastrointestinal pathology reporting (555, 556). The use of automated systems, as in the present study, can increase the objectivity and reproducibility of quantitative-based assessment (445, 464, 557). Therefore, it is not surprising that automated assessment of biopsies had greater discriminatory value than manual assessment with respect to predicting both the T-lymphocyte density of surgically resected specimens and prognosis. Such systems, however, have been criticised with respect to differentiating between tumour and non-tumour tissue as well as discriminating between true and background staining (445, 557). To allow for these limitations in the present study, all sections were marked with an appropriate region of interest. Although not allowing for full automation of the process, this did however ensure that only tumour-containing regions and those with appropriate staining were considered for analysis therefore reducing the possibility of software or algorithm-based error.

In addition to allowing for pre-operative assessment of the tumour microenvironment, biopsy-based assessment was shown to have prognostic value independent of TNM staging. Using the combination of biopsy TSP and T-lymphocyte density alone, it was possible to stratify five-year survival from 88% to 49%. This is strikingly similar to the prognostic value of full section analysis of the tumour microenvironment using the conventional GMS or a derived GMS utilising T-lymphocyte density. Although it is not expected that biopsy-based assessment of the tumour microenvironment will replace full

section analysis following potentially curative surgery, it may however have a role in the treatment of patients not undergoing surgical resection. For example, it would be of considerable interest to investigate whether biopsy assessment of the tumour microenvironment may stratify survival of patients with metastatic colorectal cancer, and how this may subsequently impact upon therapeutic options.

The present study is limited by its lack of an automated assessment of the tumour-associated stroma. As such it was not possible to fully automate assessment of the tumour microenvironment as measured using biopsies or surgically resected specimens. This was not performed as automated digital pathology systems may not always differentiate between tumour and non-tumour tissue on H&E-stained sections (557). Given that the tumour-associated stroma is predominantly comprised of mesenchymal myofibroblasts, immunohistochemistry staining for an associated protein, such as α-smooth muscle actin (558), may facilitate future attempt at automation. Furthermore, modern digital pathology systems can be readily 'educated' to differentiate between different tumour compartments. In addition, prospective validation of the above described biopsy-based assessments of the tumour microenvironment are required before they can be routinely adopted in clinical and research practice.

In conclusion, the results of the present study suggest that assessment and staging of the tumour microenvironment of patients undergoing resection of colorectal cancer is feasible using colonoscopic biopsies. This will allow for appropriate stratification of patients entering clinical trials targeting the colorectal cancer tumour microenvironment.

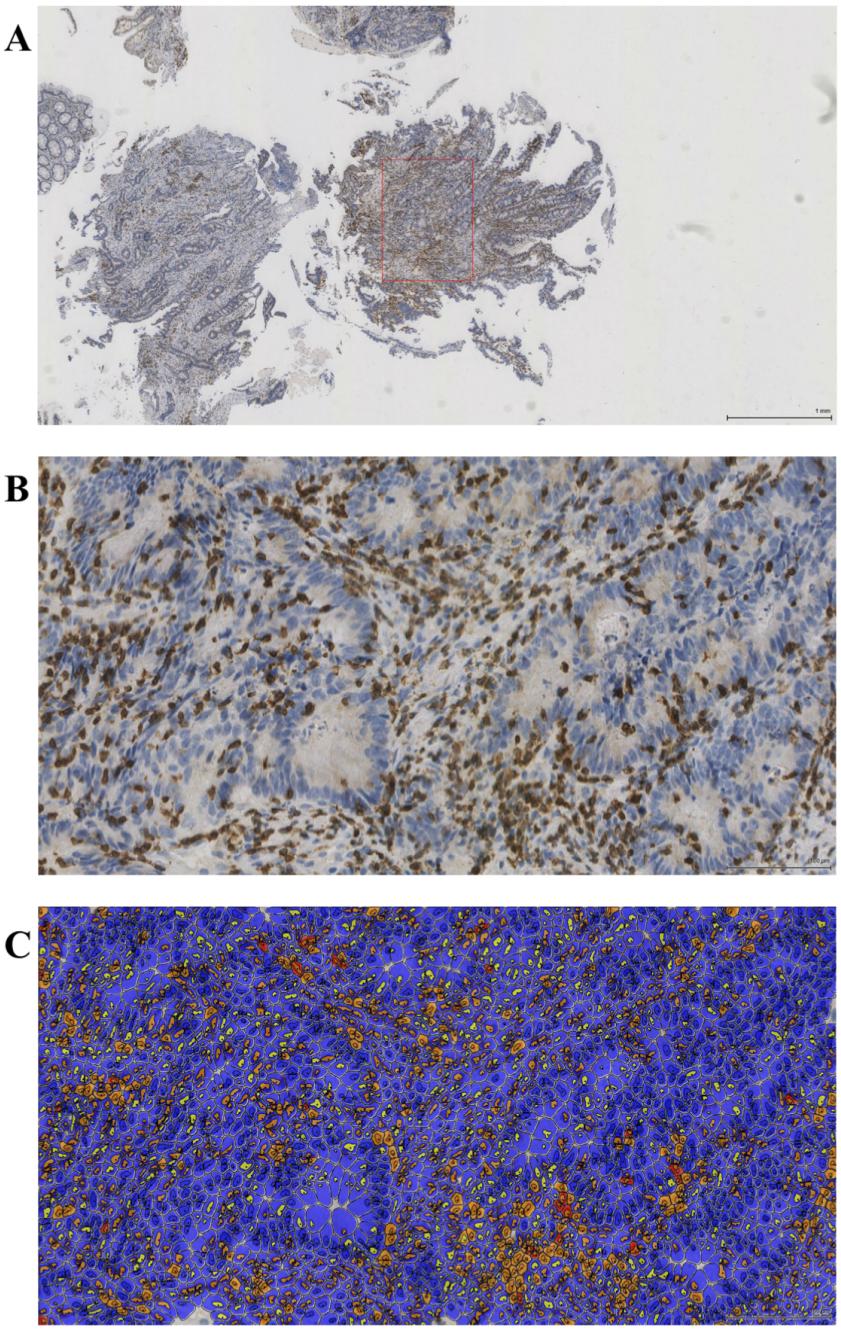


Figure 11.1 Assessment of T-lymphocyte density using colonoscopic biopsies of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** biopsy specimen stained for $CD3^+$ T-lymphocytes at x20 magnification. **(B)** the same specimen, typical of a high T-lymphocyte density, at x100 magnification, and **(C)** the same specimen analysed using automated assessment with' *Tissue IA Optimiser'* and *'Measure stained cells algorithm*' in Tissue Image Analysis plugin for Slidepath at x100 magnification. Orange and red staining signifies $CD3^+$ T-lymphocytes, whereas blue and purple staining signifies non-stained cells

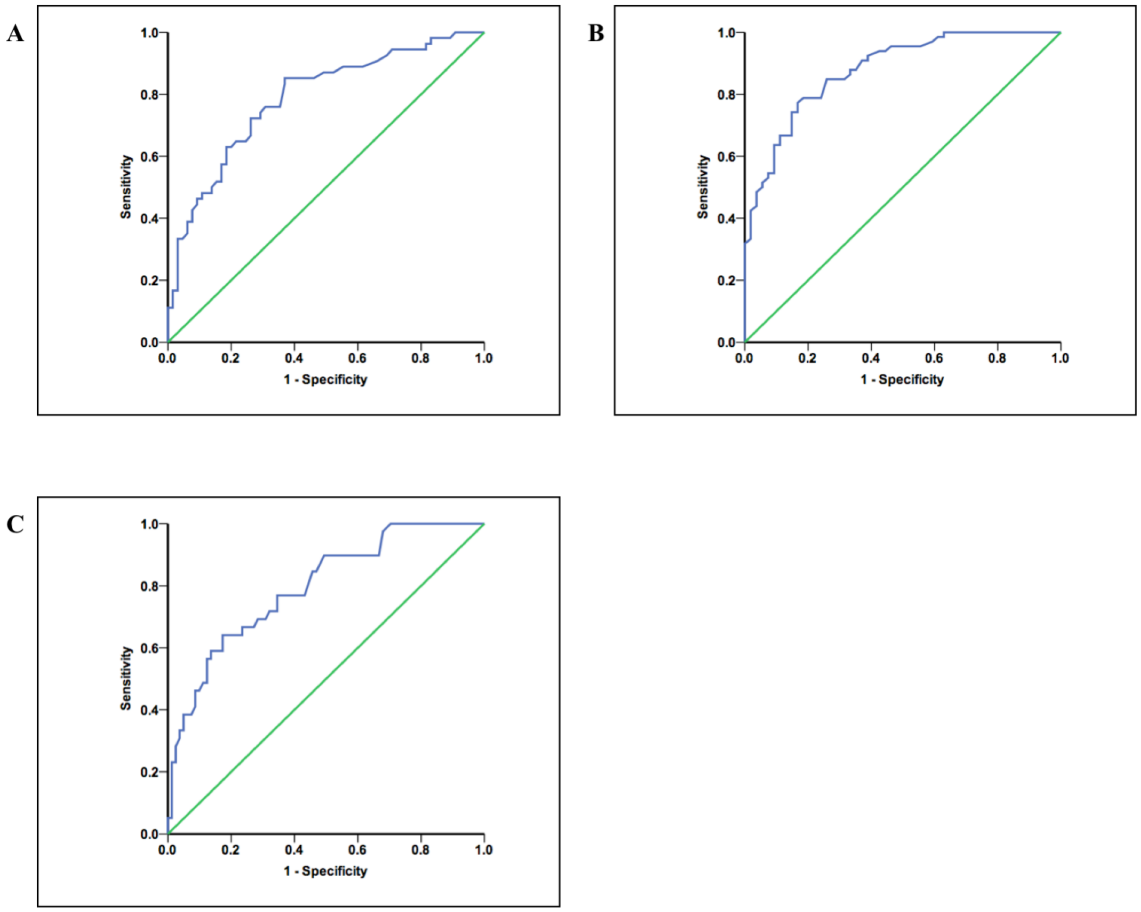


Figure 11.2 Receiver operator characteristic curves comparing automated assessment of T-lymphocyte density within different regions of surgically resected specimens from patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** invasive margin (AUC 0.787, *P*<0.001), **(B)** tumour stroma (AUC 0.879, *P*<0.001), and **(C)** tumour cancer cell nests (AUC 0.794, *P*<0.001)

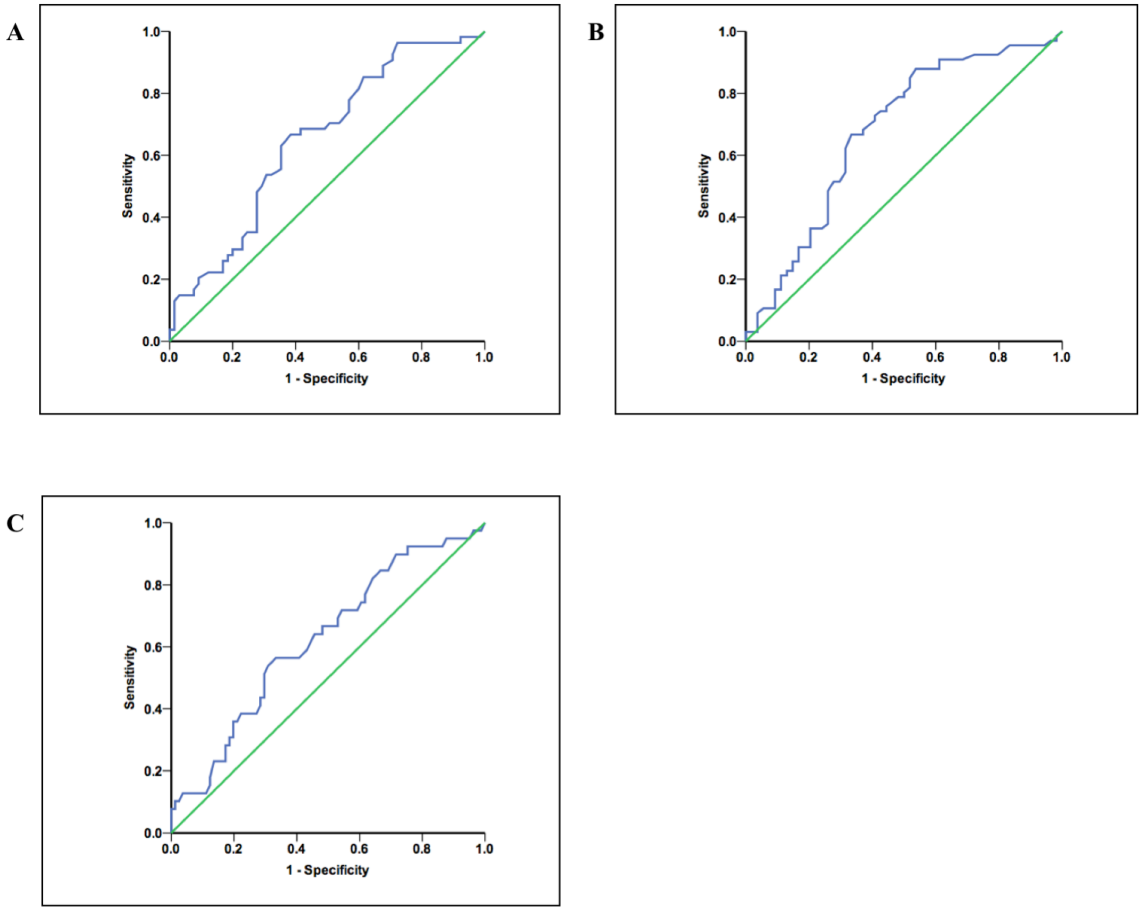
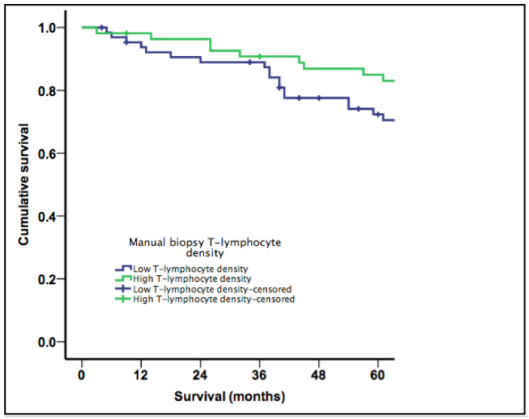
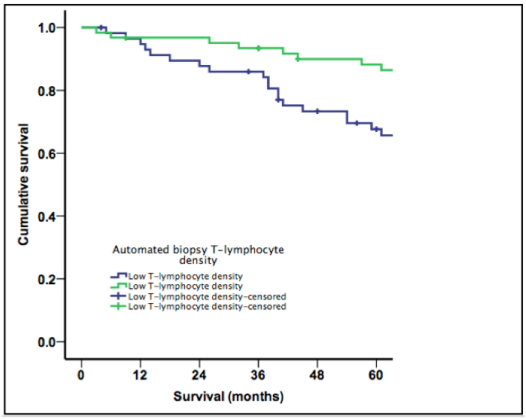


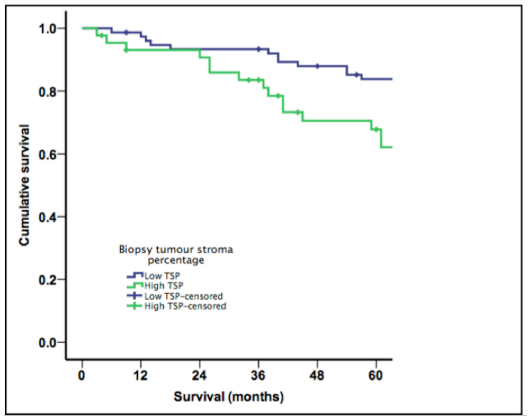
Figure 11.3 Receiver operator characteristic curves comparing automated assessment of colonoscopic biopsy T-lymphocyte density and manual assessment of T-lymphocyte density within different regions of surgically resected specimens from patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** invasive margin (AUC 0.651, *P*=0.005), **(B)** tumour stroma (AUC 0.677, *P*=0.001), and **(C)** tumour cancer cell nests (AUC 0.622, *P*=0.030)



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
High density	55	53	52	49	45	44
Low density	65	60	57	55	46	41

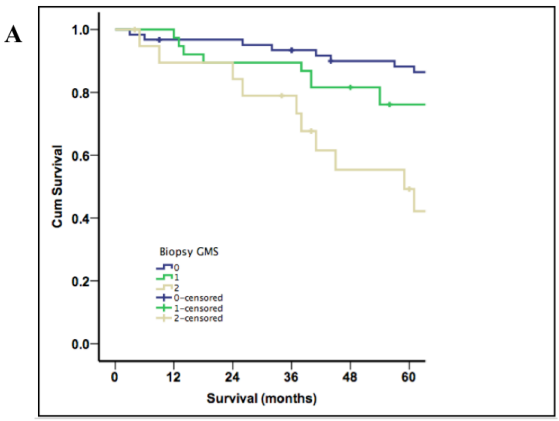


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
High density	62	58	58	56	51	50
Low density	58	55	51	48	40	35

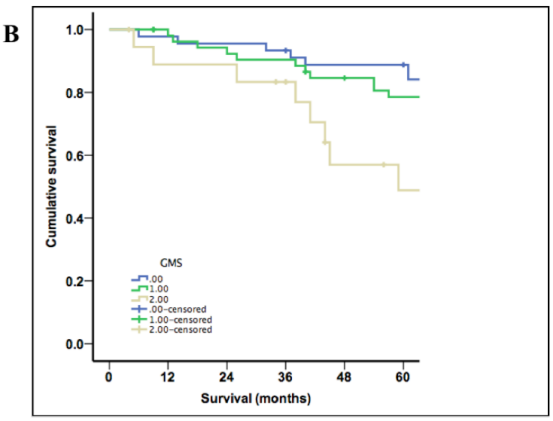


Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
Low	76	74	70	70	65	60
High	44	39	39	34	26	25

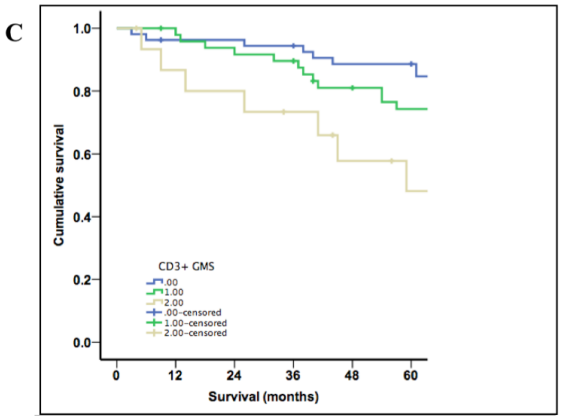
Figure 11.4 The relationship between colonoscopic biopsy-based assessment of the tumour microenvironment and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** manual assessment of T-lymphocyte density (P=0.120), **(B)** automated assessment of T-lymphocyte density (P=0.007), and **(C)** tumour stroma percentage (P=0.005). All P-values calculated using log-rank analysis



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
GMS 0	62	58	58	56	51	50
GMS 1	38	38	34	34	31	27
GMS 2	20	17	17	14	9	8



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
GMS 0	45	44	43	42	39	39
GMS 1	54	52	49	47	43	39
GMS 2	19	16	§6	14	8	6



Number at risk	Year 0	Year 1	Year 2	Year 3	Year 4	Year 5
GMS 0	54	51	51	50	46	46
GMS 1	49	48	45	43	37	33
GMS 2	16	13	12	10	7	5

Figure 11.5 The relationship between biopsy derived and full section assessment of the Glasgow Microenvironment Score and cancer-specific survival of patients undergoing potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy. **(A)** biopsy Glasgow Microenvironment Score (P<0.001), **(B)** conventional Glasgow Microenvironment Score (P=0.029), and **(C)** T-lymphocyte-derived Glasgow Microenvironment Score (P=0.019). All P-values calculated using log-rank analysis

Table 11.1 Clinicopathological characteristics of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

		All
Clinicopathological Characteristics		***n*=120 (%)**
Host characteristics		
Age		
	<65	44 (36)
	65-74	38 (32)
	>75	38 (32)
Sex		
	Female	56 (47)
	Male	64 (53)
Adjuvant therapy		
	No	83 (69)
	Yes	37 (31)
Tumour characteristics		
Tumour site		
	Colon	82 (68)
	Rectum	38 (32)
T stage		
	1/2	12 (10)
	3	75 (62)
	4	33 (28)
N stage		
	0	61 (51)
	1	46 (38)
	2	13 (11)
TNM stage		
	I	6 (5)
	II	55 (46)
	III	59 (49)
Tumour differentiation		
	Mod/well	115 (96)
	Poor	5 (4)
Venous invasion		
	No	80 (67)
	Yes	40 (33)
Margin involvement		
	No	113 (94)
	Yes	7 (6)
Peritoneal involvement		
	No	87 (72)
	Yes	33 (28)
Tumour perforation		
	No	117 (97)
	Yes	3 (3)
$CD3^+$ invasive margin density		
	Low	65 (55)
	High	54 (45)
$CD3^+$ stromal density		
	Low	54 (45)
	High	66 (55)
$CD3^+$ cancer cell nest density		
	Low	81 (67)
	High	39 (33)
Tumour stroma percentage		
	Low	93 (77)
	High	27 (23)

Table 11.2 The relationship between colonoscopic biopsy and full section manual assessment of the tumour microenvironment of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy
Data analysed using χ^2 analysis for linear trend.

		Colonoscopic biopsy		
		T-lymphocyte density		
Surgically resected specimen		**Low (*n*=65) (%)**	**High (*n*=55) (%)**	***P***
Invasive margin T-lymphocyte density				0.006
	Low	43 (66)	22 (41)	
	High	22 (34)	32 (59)	
Stromal T-lymphocyte density				0.313
	Low	32 (49)	22 (40)	
	High	33 (51)	33 (60)	
Cancer cell nest T-lymphocyte density				0.046
	Low	49 (75)	32 (58)	
	High	16 (25)	23 (42)	
		Tumour stroma percentage		
		Low (*n*=55) (%)	**High (*n*=65) (%)**	***P***
Tumour stroma percentage				0.001
	Low	66 (87)	27 (61)	
	High	10 (13)	17 (39)	

Table 11.2 displays the relationship between manual assessment of biopsy T-lymphocyte density and TSP and full section manual assessment of T-lymphocyte density and TSP. Biopsy assessment of T-lymphocyte density was associated with density within the invasive margin and cancer cell nests of full sections (both $P<0.05$) but not within the stroma. Biopsy TSP was associated with full section TSP ($P=0.001$).

Table 11.3 The relationship between automated and manual assessment of T-lymphocyte density in surgically resected specimens of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

		Automated assessment		
Manual assessment		**Median h-score (IQR)**	***P***	**Area under the curve (95% CI)**
Invasive margin T-lymphocyte density			<0.001	0.787 (0.704-0.869)
	Low	16 (11-25)		
	High	33 (22-52)		
Stromal T-lymphocyte density			<0.001	0.879 (0.820-0.938)
	Low	13 (10-19)		
	High	34 (23-51)		
Cancer cell nest T-lymphocyte density			<0.001	0.794 (0.710-0.878)
	Low	17 (11-28)		
	High	38 (23-63)		

Continuous data analysed using Mann-Whitney U test. Area under the curve calculated using receiver-operator character curves. IQR – inter-quartile range, 95% CI – 95% confidence interval.

Table 11.3displays the relationship between automated and manual assessment of T-lymphocyte density within full sections to ensure that both methods are comparable. Automated assessment of T-lymphocyte density was associated with manual assessment of density within the invasive margin, cancer cell nests and stroma of full sections (all $P<0.001$).

Table 11.4 The relationship between automated assessment of the tumour inflammatory cell infiltrate using colonoscopic biopsies and manual assessment of the inflammatory cell infiltrate using surgically resected specimens of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

		Automated assessment		
Manual assessment		**Median H-score (IQR)**	***P***	**Area under the curve (95% CI)**
Invasive margin $CD3^+$ density			0.005	0.651 (0.553-0.749)
	Low	48 (23-78)		
	High	65 (40-93)		
Stromal $CD3^+$ density			0.001	0.677 (0.578-0.776)
	Low	39 (23-74)		
	High	64 (45-93)		
Cancer cell nest $CD3^+$ density			0.030	0.622 (0.516-0.728)
	Low	52 (31-78)		
	High	66 (39-93)		

Continuous data analysed using Mann-Whitney U test. Area under the curve calculated using receiver-operator character curves. IQR – inter-quartile range, 95% CI – 95% confidence interval.

Table 11.4 displays the relationship between automated assessment of biopsy T-lymphocyte density and manual assessment within full sections. Automated assessment of biopsy T-lymphocyte density was associated with manual assessment of density within the invasive margin, cancer cell nests and stroma of full sections (all $P<0.05$).

Table 11.5 The relationship between automated assessment of T-lymphocyte density in colonoscopic biopsies and manual assessment of T-lymphocyte density in surgically resected specimens of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

		Colonoscopic biopsy Automated assessment		
Surgically resected specimen Manual assessment		**Low (*n*=58) (%)**	**High (*n*=62) (%)**	***P***
Invasive margin T-lymphocyte density				0.002
	Low	40 (69)	25 (41)	
	High	18 (31)	36 (59)	
Stromal T-lymphocyte density				<0.001
	Low	36 (62)	19 (29)	
	High	22 (38)	44 (71)	
Cancer cell nest T-lymphocyte density				0.060
	Low	44 (76)	37 (60)	
	High	14 (24)	25 (40)	

Data analysed using χ^2 analysis for linear trend.

Table 11.6 The relationship between colonoscopic biopsy-derived assessment of the tumour microenvironment, clinicopathological characteristics and cancer-specific survival of patients undergoing elective, potentially curative resection of stage I-III colorectal cancer without neoadjuvant therapy

	Cancer-specific survival			
Clinicopathological characteristics	**Univariate analysis**	***P***	**Multivariate analysis**	***P***
Age (<65/ 65-74/ >75)	1.16 (0.77-1.75)	0.488	-	-
Sex (Female/ male)	1.30 (0.66-2.59)	0.442	-	-
Adjuvant therapy (No/ yes)	1.29 (0.64-2.61)	0.480	-	-
Tumour site (Colon/ rectum)	1.66 (0.83-3.33)	0.150	-	-
TNM stage (I/ II/ III)	2.64 (1.33-5.24)	0.005	2.42 (1.19-4.94)	0.015
Tumour differentiation (Mod-well/ poor)	1.53 (0.37-6.37)	0.562	-	-
Venous invasion (No/ yes)	3.32 (1.67-6.61)	0.001	2.24 (1.09-4.63)	0.029
Margin involvement (No/ yes)	3.86 (1.34-11.10)	0.012	2.93 (0.97-8.91)	0.058
Peritoneal involvement (No/ yes)	1.76 (0.88-3.51)	0.112	-	-
Tumour perforation (No/ yes)	1.79 (0.27-14.48)	0.505	-	-
Manual biopsy T-lymphocyte density (Low/ high)	0.58 (0.29-1.17)	0.125	-	-
Automated biopsy T-lymphocyte density (Low/ high)	0.39 (0.19-0.80)	0.010	0.44 (0.21-0.92)	0.030
Biopsy tumour stroma percentage (Low/ high)	2.56 (1.30-5.04)	0.007	2.88 (1.44-5.75)	0.003

Data analysed using Cox proportional hazards regression to calculate hazard ratios and 95% confidence intervals. Backwards conditional method used for multivariate analysis.

12 Conclusions

At the beginning of this period of research, it was clear that the present TNM-based staging of patients with colorectal cancer is suboptimal. Although staging and need for adjuvant therapy are primarily based on the presence of lymph node metastases, a significant proportion of patients with lymph node negative disease may subsequently die prematurely from their disease. Conversely, a proportion of patients with lymph node positive disease have survival comparable to those with earlier stage disease. A number of pathological and molecular characteristics identifying patients at high risk have been defined, however there remains a need to identify other factors which may aid in risk stratification and decision making regarding treatment. One approach is to consider the local and systemic environment, encompassing both host inflammatory responses and the tumour microenvironment. In addition to identifying patients at high risk of recurrence, the host inflammatory response to cancer provides an attractive therapeutic target. Indeed, conventional anti-inflammatory drugs, such as aspirin and NSAIDs, have been associated with improved outcomes in patients with colorectal cancer, potentially through modulation of tumour-associated inflammation (Chapter 1).

In spite of a significant body of work supporting the role of tumour-associated inflammation as being integral to disease progression and an important determinant of outcome, several questions remained. First, how measures of the systemic inflammatory response, such as the mGPS may be utilised alongside present TNM-based staging, and how this may be applied across different populations from distinct geographical regions, was unclear. Similarly, how more comprehensive assessment of the tumour microenvironment, encompassing not only the tumour inflammatory cell infiltrate but also the tumour-associated stroma, may be used to determine prognosis was not known. Furthermore, the underlying tumour-based characteristics which may determine these local and systemic responses remained to be fully investigated. Finally, it was not clear whether

the anti-neoplastic effects of commonly prescribed drugs such as aspirin and statins is in part mediated by favourable effects on host inflammatory responses.

This thesis started with a comparison of the relative prognostic value of assessment of the pre-operative systemic inflammatory response and TNM-based staging in patients undergoing potentially curative resection of stage I-III colorectal cancer (Chapter 2). The mGPS, one of the most widely reported systemic inflammatory scores in patients with cancer, was utilised. It was shown that the mGPS held prognostic value independent of TNM staging and other pathological characteristics associated with high risk disease. Furthermore, in patients with stage III colon cancer, the presence of a systemic inflammatory response appeared to abrogate any survival benefit from adjuvant 5-FU-based chemotherapy. Although patients with colorectal cancer and systemic inflammation are at increased risk of recurrence and death, the results of this Chapter would suggest that novel therapeutic agents, most likely targeting the inflammatory response, rather than conventional chemotherapy may be needed in this specific patient group. Further work in this field should be performed to investigate the relationship between systemic inflammation and response to adjuvant chemotherapy as well as anti-inflammatory drugs, potentially by retrospective analysis of clinical trial data.

In Chapter 3, it was shown that systemic inflammatory profiles differ between populations from distinct geographical locations; in the present case, populations of patients with colorectal cancer from the West of Scotland and Japan. Although associated with similar patient and tumour characteristics in both populations, even after controlling for these factors patients from Scotland were more likely to be systemically inflamed. The underlying reason why distinct populations may differ in their inflammatory profile is unclear, however it was hypothesised that this may be attributable to uncontrolled factors, such as comorbidity and obesity. However, the mGPS showed prognostic value in both populations. Indeed, given its differing prevalence across populations, it is clear that

measures of the systemic inflammatory response should be reported routinely, particularly in the context of outcome data. The present results could be confirmed retrospectively utilising international clinical trial data. Furthermore, studies of the relationship between systemic inflammation and cancer outcome could be performed in regions with large migrant populations to ascertain if the differences observed are indeed secondary to host or environmental factors.

In Chapter 4 the relationship between the tumour-associated stroma, other components of the tumour microenvironment, such as tumour necrosis and the local inflammatory cell infiltrate, and survival was examined. Using the TSP, an H&E-based assessment of the extent of stromal infiltration, it was found that a high proportion of stroma was associated with adverse tumour characteristics, such as advanced T stage, an infiltrative invasive margin, and loss of the tumour-infiltrating inflammatory cell infiltrate. Of interest, an expanded stroma was inversely associated with the presence of tumour necrosis, suggesting a pertinent role in protecting against hypoxia. Despite such associations, a high TSP remained independently associated with reduced survival of patients with colorectal cancer, validating previous work by Mesker and colleagues. Taken together, these results further confirm the stroma as an important contributor towards a tumour-supporting microenvironment and as a potential therapeutic target. Future work could refine the prognostic value of the tumour-associated stroma by examining such characteristics as stromal and collagen maturity in addition to TSP. Furthermore, chemotherapeutics which target the stroma or overcome the stroma as a barrier to effective tumour cell targeting, have been utilised in other tumour types. On such example is nab-paclitaxel in patients with pancreatic ductal adenocarcinoma (559). Whether a similar approach may translate in to an effective treatment strategy in patients with colorectal cancer and a high TSP would be of interest.

In Chapter 5, the role of MMR deficiency as a determinant of host local and systemic inflammatory responses was examined. Although the present results confirmed the relationship between MMR deficiency and the presence of a conspicuous inflammatory cell infiltrate, it was observed, for the first time, that patients with MMR deficient colorectal cancer had elevated systemic inflammatory responses as measured by the mGPS and the NPS. Although paradoxical, given the opposing prognostic effects of MMR deficiency and elevated systemic inflammatory responses, the present results could be explained by a number of potential mechanisms. As immune checkpoint activation is recognised to counterbalance the anti-tumour immune infiltrate in MMR deficient tumours, one potential hypothesis is that the systemic inflammatory response may represent a common upstream precursor of both phenomena, for example, the JAK/STAT3 pathway.

The results of Chapter 5 also confirm the prognostic value of local and systemic inflammatory responses independent of MMR/ MSI status. Indeed, they provide further rationale for the assessment of inflammatory responses in addition to MMR/ MSI status in patients with colorectal cancer. In keeping with this, the prognostic value of the local inflammatory cell infiltrate independent of MSI status has recently been confirmed in a large population-based case-control study of over 2000 patients (560). The relationship between MMR status, systemic inflammatory responses and outcome however await confirmation in a larger population than that presently studied. Furthermore, whether local and systemic inflammatory responses remain prognostic independent of more comprehensive genetic and molecular characterisation remains to be determined.

In Chapter 6, the relationship between the IL-6/JAK/STAT3 signal transduction pathway, local and systemic inflammatory responses and outcome of patients with colorectal cancer was examined. Activation, as measured by tumour cell STAT3 expression, was associated with adverse inflammatory responses. Despite STAT3 expression being associated with reduced survival on univariate analysis, it was not independent of pathological

characteristics nor local and systemic inflammatory responses. It is likely that the local and systemic environment is defined by a number of pathways, with JAK/STAT3 activation being just one mechanism by which the tumour deregulates host inflammatory responses. Indeed, although the JAK/STAT3 pathway may be one potential therapeutic target, future work is needed to investigate and compare other pro-inflammatory pathways, such as NF-κB. Furthermore, whether assessment of inflammatory responses, and in particular the mGPS, may identify patients likely to benefit from inhibitors of JAK/STAT3 and other inflammatory pathways, remains to be investigated.

Given the independent prognostic value of the tumour-associated stroma and inflammatory cell infiltrate, Chapter 7 aimed to investigate the clinical utility of their combined assessment. The prognostic value of the stroma was subordinate to the local inflammatory cell infiltrate, however combined assessment stratified survival greater than either measure alone. A combined score based on these characteristics, termed the Glasgow Microenvironment Score, was simple to perform and relied on routine specimens with no additional costs. Therefore, it can be readily validated by independent groups. Although observational only, the results also give further potential insight into the natural history of the tumour microenvironment; it would appear that it is loss of the anti-tumour immune response which is the early initiator of a supportive tumour microenvironment, with tumour stroma expansion occurring at a later point. Although speculative, this hypothesis may be readily examined in larger clinical cohort studies encompassing patients with early stage disease.

The definition of T stage has remained relatively stable since initially being described by Dukes. As such, Chapter 8 aimed to examine how increasing T stage, as a marker of tumour invasiveness, related to both the local and systemic environment. As would be expected, as tumour invasiveness increased, the tumour microenvironment became more supportive, with loss of the inflammatory cell infiltrate as measured by both Immunoscore

and KM grade, an increase in TSP and the presence of an infiltrative invasive margin. Furthermore, systemic inflammatory responses increased with increasing T stage. Of interest however, the development of these characteristics appeared to follow a stepwise pattern, with loss of the immune infiltrate occurring at an earlier T stage and development of a high TSP, infiltrative margin and an elevated systemic inflammatory response occurring later. Furthermore, the GMS, Immunoscore and mGPS, appeared to have similar if not greater prognostic value compared to lymph node status when examined in the context of patients with T3 disease. Although only providing a cross-sectional view of the relationship between tumour invasiveness and such characteristics, these results further support the hypothesis that loss of the local inflammatory cell infiltrate is an important initiating step in the development of a tumour-favouring environment at both the local and systemic level. In addition, they support the routine assessment of both local and systemic inflammatory responses in patients with colorectal cancer. It is clear however that further work is required in this area, particularly in earlier stage disease to confirm the prognostic value of assessment of the local and systemic environment.

Chapter 9 aimed to examine the clinical utility of two differing approaches to assessment of the local inflammatory cell infiltrate, namely the KM grade and Immunoscore. It was found that the Immunoscore stratified survival to a greater extent than KM grade; indeed, it was possible to stratify survival of patients with both a low and high KM grade using the Immunoscore. From these findings it was hypothesised that in those patients with a discordance between measures of the generalised and T-lymphocytic infiltrate (i.e. high KM grade but low Immunoscore), the peritumoural inflammatory infiltrate represented other components of the host cellular immune response, most likely innate immune cells such as neutrophils and macrophages, with an adverse effect on outcome. It was of interest however, that the TSP was able to stratify survival of patients with both a low Immunoscore and KM grade. Indeed, this would further support the routine assessment of

TSP in addition to the local inflammatory cell infiltrate in patients with colorectal cancer. The results of this Chapter further support the efforts of a recent international collaborative, which has validated the Immunoscore as a stage-independent prognostic factor in patients with colorectal cancer (561). It is clear however that further work is required to validate assessment of the TSP as an additional prognostic factor, particularly in combination with assessment of the inflammatory cell infiltrate.

Although aspirin and statins are reported to have a potential anti-inflammatory effect in patients with cancer, it is not clear how their routine use at time of diagnosis affects markers of the systemic inflammatory response. In Chapter 10, it was found that pre-operative use of low-dose aspirin, but not statins, was associated with a lower pre-operative mGPS in patients undergoing resection of colorectal cancer, suggesting a beneficial effect on the systemic inflammatory response in patients with colorectal cancer. Survival data was immature, however aspirin users appeared to have poorer survival than non-aspirin users. This is likely to reflect the underlying reason for aspirin use in this cohort, as all patients received aspirin for cardiovascular risk modification. However, the relationship between aspirin use and a lower mGPS was surprising, as comorbidity burden is associated with elevated systemic inflammatory responses. Indeed, the true anti-inflammatory effect of aspirin in patients with colorectal cancer may be underestimated in this cohort. Further studies are required to examine the relationship between aspirin and NSAID use, systemic inflammatory responses and outcome of patients with colorectal cancer. This could be performed in the context of currently recruiting trials of adjuvant aspirin therapy. Furthermore, investigation of the relationship between aspirin and NSAIDs and characteristics of the tumour microenvironment are required. Retrospective assessment of the tumour microenvironment in archived tumour tissue from the cohort studied in this Chapter would be a logical starting point for ongoing work in this field.

Finally, in Chapter 11, the feasibility of pre-operative assessment of the tumour microenvironment, using colonoscopic biopsy specimens, was examined. It was found that both the tumour inflammatory cell infiltrate, as measured by T-lymphocyte density, and the TSP could be measured using biopsy specimens. Furthermore, the reliability of the former was improved by use of automated digital pathology. A biopsy GMS derived from these measures was independently associated with survival. Although it would not be expected that biopsy-based assessment would replace more comprehensive assessment using surgically resected specimens, these results do raise two interesting concepts for further investigation. Firstly, it would allow for assessment of the primary tumour microenvironment in patients with metastatic disease or those who are not candidates for curative resection. Secondly, it would potentially allow for pre-operative staging of the tumour microenvironment, therefore creating a window of opportunity for neoadjuvant therapy directed at the tumour microenvironment. Indeed, whether aspirin and NSAIDs, or other novel therapeutic agents such as JAK/STAT3 inhibitors may be of use in this setting would be of considerable interest.

In summary, the present thesis suggests that the local and systemic environment create a supportive environment which promotes continued tumour growth and dissemination to the detriment of the patient. In addition to determining prognosis of patients with colorectal cancer, measurement of these characteristics may yield potential therapeutic targets. The above work confirms that assessment of the systemic inflammatory response, using routinely available prognostic scores such as the mGPS, complements TNM staging to identify patients with otherwise "low risk" disease at high risk of recurrence. Conversely, it is also possible to "downstage" patients deemed high risk based on lymph node involvement alone. Furthermore, the mGPS appears to be applicable internationally, further supporting its routine reporting. A similar approach may be taken with respect to the tumour microenvironment, with combined assessment of both the tumour inflammatory

cell infiltrate and tumour-associated stroma having greater prognostic value than either measure alone. Although the GMS described herein provides an attractive concept because of its reliance on routine specimens and relative simplicity, it may well be that more refined measures of the local inflammatory response, such as the Immunoscore, provide a more reliable measure of host anti-tumour immunity. However, there remains a need to examine other components of the tumour microenvironment, such as tumour cell budding and innate immune cell infiltration, as potential adjuncts to a comprehensive tumour microenvironment-based score and as potential therapeutic targets. Such work may increase our insights into the development of the tumour microenvironment.

The work presented in this thesis suggest that several factors, pertaining to not only the host but also the tumour, may determine characteristics of the local and systemic environment (Figure 12.1). Indeed, although some of these factors, such as MMR status, may be tumour cell-intrinsic and therefor non-modifiable, targeting intracellular signalling pathways presents one potential therapeutic option which may be further investigated. However, the work presented herein suggests that the local and systemic environment are shaped by a number of different pathways, and it may be that the predominant pathways differ between patients. Indeed, whether JAK/STAT3 is the optimal target or whether other pathways may have greater impact on cancer-association inflammation and survival remains to be determined. Further work, exploring the molecular characteristics associated with each of the phenotypic features examined in this thesis will hopefully identify potential druggable targets which may be utilised in future clinical practice. Irrespective, the work presented suggests that pre-operative assessment at the local and systemic level is feasible in this patient group prior to resection; this may aid in the identification of suitable candidates for enrolment in clinical trials targeting such targets and the local and systemic environment in general.

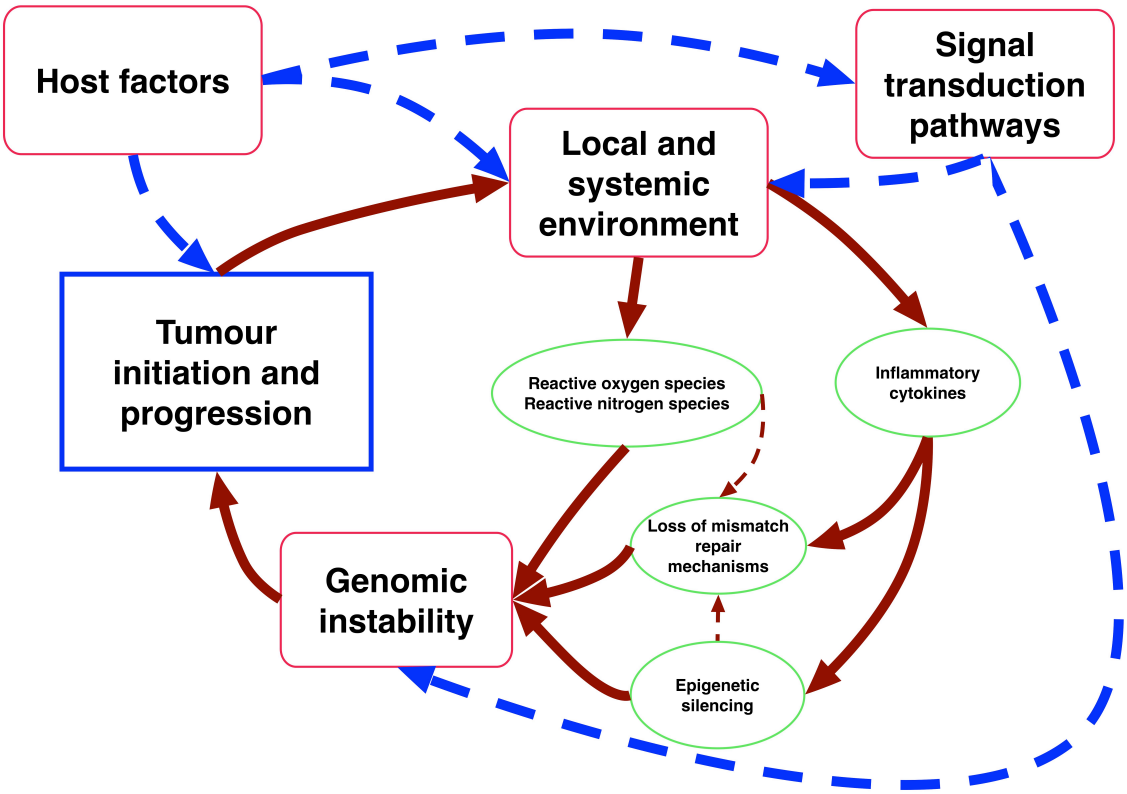


Figure 12.1 The relationship between the local and systemic environment, host factors, signal transduction pathway activation and genomic instability and tumour initiation and progression

In summary, this thesis has aimed to address the two hypotheses stated in Chapter 1.8. The work presented supports the role of the local and systemic environment, encompassing the tumour microenvironment and systemic inflammatory responses, as potential adjuncts in the staging of patients undergoing potentially curative resection of stage I-III colorectal cancer. Furthermore, a number of tumour and host factors determine the local and systemic environment. Although some may be tumour-cell intrinsic and therefore non-modifiable, others, including inflammatory signal transduction pathways, may provide attractive therapeutic targets which may reduce risk of recurrence and increase survival of patients with colorectal cancer.

13 References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61(2):69-90.

2. Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer. 2010;46(4):765-81.

3. Luengo-Fernandez R, Leal J, Gray A, Sullivan R. Economic burden of cancer across the European Union: a population-based cost analysis. Lancet Oncol. 2013;14(12):1165-74.

4. Karim-Kos HE, de Vries E, Soerjomataram I, Lemmens V, Siesling S, Coebergh JW. Recent trends of cancer in Europe: a combined approach of incidence, survival and mortality for 17 cancer sites since the 1990s. Eur J Cancer. 2008;44(10):1345-89.

5. UK CR. Bowel cancer incidence statistics 2013 [updated 11/06/2014. Available from: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bowel/incidence/ - By2.

6. Network CRUaNCI. Cancer by deprivation in England: Incidence, 1996-2010, Mortality, 1997-2011. London: NCIN; 2014.

7. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer. 2013;49(6):1374-403.

8. UK CR. Bowel cancer mortality statistics Cancer Research UK: Cancer Research UK; 2013 [updated April 3, 2013. Available from: http://info.cancerresearchuk.org/cancerstats/types/bowel/mortality.

9. Worthley DL, Whitehall VL, Spring KJ, Leggett BA. Colorectal carcinogenesis: road maps to cancer. World J Gastroenterol. 2007;13(28):3784-91.

10. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988;319(9):525-32.

11. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer. 2009;9(7):489-99.

12. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61(5):759-67.

13. Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg. 2002;89(7):845-60.

14. Takayama T, Ohi M, Hayashi T, Miyanishi K, Nobuoka A, Nakajima T, et al. Analysis of K-ras, APC, and beta-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. Gastroenterology. 2001;121(3):599-611.
15. Rajagopalan H, Lengauer C. Aneuploidy and cancer. Nature. 2004;432(7015):338-41.
16. Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology. 2010;138(6):2073-87 e3.
17. Ward R, Meagher A, Tomlinson I, O'Connor T, Norrie M, Wu R, et al. Microsatellite instability and the clinicopathological features of sporadic colorectal cancer. Gut. 2001;48(6):821-9.
18. Jass JR, Whitehall VL, Young J, Leggett BA. Emerging concepts in colorectal neoplasia. Gastroenterology. 2002;123(3):862-76.
19. Greenson JK, Huang SC, Herron C, Moreno V, Bonner JD, Tomsho LP, et al. Pathologic predictors of microsatellite instability in colorectal cancer. Am J Surg Pathol. 2009;33(1):126-33.
20. Smyrk TC, Watson P, Kaul K, Lynch HT. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer. 2001;91(12):2417-22.
21. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998;58(22):5248-57.
22. Arends M, Ibrahim M, Happerfield L, Frayling I, Miller K. Interpretation of immunohistochemical analysis of mismatch repair (MMR) protein expression in tissue sections for investigation of suspected Lynch/Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome. UK NEQAS ICC & ISH Recommendations. 2008;1.
23. Jass JR. Serrated adenoma of the colorectum and the DNA-methylator phenotype. Nat Clin Pract Oncol. 2005;2(8):398-405.
24. Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner GJ, Weisenberger DJ, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut. 2006;55(7):1000-6.
25. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006;38(7):787-93.

26. Lynch HT, Lynch JF, Lynch PM, Attard T. Hereditary colorectal cancer syndromes: molecular genetics, genetic counseling, diagnosis and management. Fam Cancer. 2008;7(1):27-39.
27. Gryfe R. Inherited colorectal cancer syndromes. Clin Colon Rectal Surg. 2009;22(4):198-208.
28. Mishra N, Hall J. Identification of patients at risk for hereditary colorectal cancer. Clin Colon Rectal Surg. 2012;25(2):67-82.
29. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. CA: a cancer journal for clinicians. 2009;59(6):366-78.
30. Staszewski J, McCall MG, Stenhouse NS. Cancer mortality in 1962-66 among Polish migrants to Australia. Br J Cancer. 1971;25(4):599-610.
31. Locke FB, King H. Cancer mortality risk among Japanese in the United States. J Natl Cancer Inst. 1980;65(5):1149-56.
32. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. Cancer Epidemiol Biomarkers Prev. 2009;18(6):1688-94.
33. Burkitt DP. Possible relationships between bowel cancer and dietary habits. Proc R Soc Med. 1971;64(9):964-5.
34. Rieger MA, Parlesak A, Pool-Zobel BL, Rechkemmer G, Bode C. A diet high in fat and meat but low in dietary fibre increases the genotoxic potential of 'faecal water'. Carcinogenesis. 1999;20(12):2311-6.
35. Bingham SA. Mechanisms and experimental and epidemiological evidence relating dietary fibre (non-starch polysaccharides) and starch to protection against large bowel cancer. Proc Nutr Soc. 1990;49(2):153-71.
36. Gonzalez CA, Riboli E. Diet and cancer prevention: Contributions from the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Eur J Cancer. 2010;46(14):2555-62.
37. Murphy N, Norat T, Ferrari P, Jenab M, Bueno-de-Mesquita B, Skeie G, et al. Dietary fibre intake and risks of cancers of the colon and rectum in the European prospective investigation into cancer and nutrition (EPIC). PloS one. 2012;7(6):e39361.
38. Aune D, Chan DS, Lau R, Vieira R, Greenwood DC, Kampman E, et al. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. BMJ. 2011;343:d6617.
39. World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washginton DC: AICR; 2007.

40. Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, et al. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. PloS one. 2011;6(6):e20456.
41. World Cancer Research Fund / American Institute for Cancer Research. Continuous Update Project Report. Food, Nutrition, Physical Activity, and the Prevention of Colorectal Cancer. 2011.
42. Hopkins MH, Owen J, Ahearn T, Fedirko V, Flanders WD, Jones DP, et al. Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a randomized, controlled clinical trial. Cancer Prev Res (Phila). 2011;4(10):1645-54.
43. Weinert BT, Timiras PS. Invited review: Theories of aging. J Appl Physiol (1985). 2003;95(4):1706-16.
44. Kanai Y, Hirohashi S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. Carcinogenesis. 2007;28(12):2434-42.
45. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Kearney J, et al. A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men. J Natl Cancer Inst. 1994;86(3):183-91.
46. Giovannucci E, Colditz GA, Stampfer MJ, Hunter D, Rosner BA, Willett WC, et al. A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. women. J Natl Cancer Inst. 1994;86(3):192-9.
47. Ji BT, Weissfeld JL, Chow WH, Huang WY, Schoen RE, Hayes RB. Tobacco smoking and colorectal hyperplastic and adenomatous polyps. Cancer Epidemiol Biomarkers Prev. 2006;15(5):897-901.
48. Larsen IK, Grotmol T, Almendingen K, Hoff G. Lifestyle as a predictor for colonic neoplasia in asymptomatic individuals. BMC Gastroenterol. 2006;6:5.
49. Lee IM, Shiroma EJ, Lobelo F, Puska P, Blair SN, Katzmarzyk PT, et al. Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. Lancet. 2012;380(9838):219-29.
50. Boyle T, Keegel T, Bull F, Heyworth J, Fritschi L. Physical activity and risks of proximal and distal colon cancers: a systematic review and meta-analysis. J Natl Cancer Inst. 2012;104(20):1548-61.
51. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut. 2001;48(4):526-35.

52. Collins PD, Mpofu C, Watson AJ, Rhodes JM. Strategies for detecting colon cancer and/or dysplasia in patients with inflammatory bowel disease. Cochrane Database Syst Rev. 2006(2):CD000279.
53. Dyson JK, Rutter MD. Colorectal cancer in inflammatory bowel disease: what is the real magnitude of the risk? World J Gastroenterol. 2012;18(29):3839-48.
54. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell. 2009;15(2):103-13.
55. Network SIG. SIGN publication no. 126. Diagnosis and management of colorectal cancer 2011.
56. Lutgens MW, Oldenburg B, Siersema PD, van Bodegraven AA, Dijkstra G, Hommes DW, et al. Colonoscopic surveillance improves survival after colorectal cancer diagnosis in inflammatory bowel disease. Br J Cancer. 2009;101(10):1671-5.
57. Doyle SL, Donohoe CL, Lysaght J, Reynolds JV. Visceral obesity, metabolic syndrome, insulin resistance and cancer. Proc Nutr Soc. 2012;71(1):181-9.
58. Ishino K, Mutoh M, Totsuka Y, Nakagama H. Metabolic syndrome: a novel high-risk state for colorectal cancer. Cancer Lett. 2013;334(1):56-61.
59. Yamamoto K, Miyata T, Nagawa H. The high prevalence of colorectal neoplasms in preoperative patients with abdominal aortic aneurysm or peripheral artery disease. Eur J Vasc Endovasc Surg. 2007;33(4):397-400.
60. Yang SY, Kim YS, Chung SJ, Song JH, Choi SY, Park MJ, et al. Association between colorectal adenoma and coronary atherosclerosis detected by CT coronary angiography in Korean men; a cross-sectional study. J Gastroenterol Hepatol. 2010;25(11):1795-9.
61. Chan AO, Jim MH, Lam KF, Morris JS, Siu DC, Tong T, et al. Prevalence of colorectal neoplasm among patients with newly diagnosed coronary artery disease. JAMA. 2007;298(12):1412-9.
62. Ohshima H, Tazawa H, Sylla BS, Sawa T. Prevention of human cancer by modulation of chronic inflammatory processes. Mutat Res. 2005;591(1-2):110-22.
63. Tsilidis KK, Branchini C, Guallar E, Helzlsouer KJ, Erlinger TP, Platz EA. C-reactive protein and colorectal cancer risk: a systematic review of prospective studies. Int J Cancer. 2008;123(5):1133-40.
64. Chan AT, Ogino S, Giovannucci EL, Fuchs CS. Inflammatory markers are associated with risk of colorectal cancer and chemopreventive response to anti-inflammatory drugs. Gastroenterology. 2011;140(3):799-808, quiz e11.

65. Prizment AE, Anderson KE, Visvanathan K, Folsom AR. Association of inflammatory markers with colorectal cancer incidence in the atherosclerosis risk in communities study. Cancer Epidemiol Biomarkers Prev. 2011;20(2):297-307.
66. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med. 1993;328(18):1313-6.
67. Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S, et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. Lancet. 2011;378(9809):2081-7.
68. Vinogradova Y, Hippisley-Cox J, Coupland C, Logan RF. Risk of colorectal cancer in patients prescribed statins, nonsteroidal anti-inflammatory drugs, and cyclooxygenase-2 inhibitors: nested case-control study. Gastroenterology. 2007;133(2):393-402.
69. Harris RE, Beebe-Donk J, Alshafie GA. Similar reductions in the risk of human colon cancer by selective and nonselective cyclooxygenase-2 (COX-2) inhibitors. BMC Cancer. 2008;8:237.
70. Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet. 2010;376(9754):1741-50.
71. Routine aspirin or nonsteroidal anti-inflammatory drugs for the primary prevention of colorectal cancer: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2007;146(5):361-4.
72. Hindler K, Cleeland CS, Rivera E, Collard CD. The role of statins in cancer therapy. Oncologist. 2006;11(3):306-15.
73. Bardou M, Barkun A, Martel M. Effect of statin therapy on colorectal cancer. Gut. 2010;59(11):1572-85.
74. Poynter JN, Gruber SB, Higgins PD, Almog R, Bonner JD, Rennert HS, et al. Statins and the risk of colorectal cancer. N Engl J Med. 2005;352(21):2184-92.
75. Hachem C, Morgan R, Johnson M, Kuebeler M, El-Serag H. Statins and the risk of colorectal carcinoma: a nested case-control study in veterans with diabetes. Am J Gastroenterol. 2009;104(5):1241-8.
76. Lakha F, Theodoratou E, Farrington SM, Tenesa A, Cetnarskyj R, Din FV, et al. Statin use and association with colorectal cancer survival and risk: case control study with prescription data linkage. BMC Cancer. 2012;12(1):487.
77. Bonovas S, Filioussi K, Flordellis CS, Sitaras NM. Statins and the risk of colorectal cancer: a meta-analysis of 18 studies involving more than 1.5 million patients. J Clin Oncol. 2007;25(23):3462-8.

78. Notarnicola M, Messa C, Pricci M, Guerra V, Altomare DF, Montemurro S, et al. Up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in left-sided human colon cancer. Anticancer Res. 2004;24(6):3837-42.
79. Zervoudakis A, Strickler HD, Park Y, Xue X, Hollenbeck A, Schatzkin A, et al. Reproductive history and risk of colorectal cancer in postmenopausal women. J Natl Cancer Inst. 2011;103(10):826-34.
80. Green J, Czanner G, Reeves G, Watson J, Wise L, Roddam A, et al. Menopausal hormone therapy and risk of gastrointestinal cancer: nested case-control study within a prospective cohort, and meta-analysis. Int J Cancer. 2012;130(10):2387-96.
81. Limsui D, Vierkant RA, Tillmans LS, Wang AH, Weisenberger DJ, Laird PW, et al. Postmenopausal hormone therapy and colorectal cancer risk by molecularly defined subtypes among older women. Gut. 2012;61(9):1299-305.
82. Simon MS, Chlebowski RT, Wactawski-Wende J, Johnson KC, Muskovitz A, Kato I, et al. Estrogen plus progestin and colorectal cancer incidence and mortality. J Clin Oncol. 2012;30(32):3983-90.
83. Gatalica Z, Torlakovic E. Pathology of the hereditary colorectal carcinoma. Fam Cancer. 2008;7(1):15-26.
84. Lynch HT, Shaw MW, Magnuson CW, Larsen AL, Krush AJ. Hereditary factors in cancer. Study of two large midwestern kindreds. Arch Intern Med. 1966;117(2):206-12.
85. Samowitz WS, Curtin K, Lin HH, Robertson MA, Schaffer D, Nichols M, et al. The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. Gastroenterology. 2001;121(4):830-8.
86. Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum. 1991;34(5):424-5.
87. Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 1999;116(6):1453-6.
88. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004;96(4):261-8.
89. Zbuk KM, Eng C. Hamartomatous polyposis syndromes. Nat Clin Pract Gastroenterol Hepatol. 2007;4(9):492-502.
90. Wirtzfeld DA, Petrelli NJ, Rodriguez-Bigas MA. Hamartomatous polyposis syndromes: molecular genetics, neoplastic risk, and surveillance recommendations. Ann Surg Oncol. 2001;8(4):319-27.

91. Playford RJ. Landscaper seeks remunerative position. Gut. 2001;48(5):594-5.
92. Thompson MR, Perera R, Senapati A, Dodds S. Predictive value of common symptom combinations in diagnosing colorectal cancer. Br J Surg. 2007;94(10):1260-5.
93. Flashman K, O'Leary DP, Senapati A, Thompson MR. The Department of Health's "two week standard" for bowel cancer: is it working? Gut. 2004;53(3):387-91.
94. McArdle CS, Hole DJ. Emergency presentation of colorectal cancer is associated with poor 5-year survival. Br J Surg. 2004;91(5):605-9.
95. Fraser CG, Digby J, McDonald PJ, Strachan JA, Carey FA, Steele RJ. Experience with a two-tier reflex gFOBT/FIT strategy in a national bowel screening programme. J Med Screen. 2012;19(1):8-13.
96. Hewitson P, Glasziou P, Irwig L, Towler B, Watson E. Screening for colorectal cancer using the faecal occult blood test, Hemoccult. Cochrane Database Syst Rev. 2007(1):CD001216.
97. Scholefield JH, Robinson MH, Mangham CM, Hardcastle JD. Screening for colorectal cancer reduces emergency admissions. Eur J Surg Oncol. 1998;24(1):47-50.
98. Steele RJ, McClements PL, Libby G, Black R, Morton C, Birrell J, et al. Results from the first three rounds of the Scottish demonstration pilot of FOBT screening for colorectal cancer. Gut. 2009;58(4):530-5.
99. Poston GJ, Tait D, O'Connell S, Bennett A, Berendse S, Guideline Development G. Diagnosis and management of colorectal cancer: summary of NICE guidance. BMJ. 2011;343:d6751.
100. Aldridge MC, Phillips RK, Hittinger R, Fry JS, Fielding LP. Influence of tumour site on presentation, management and subsequent outcome in large bowel cancer. Br J Surg. 1986;73(8):663-70.
101. ACPGBI. Guidelines for the management of bowel cancer London2007 [cited 2016. 3rd:[Available from: http://www.acpgbi.org.uk/content/uploads/2007-CC-Management-Guidelines.pdf.
102. PN W, D B. CT colongraphy: what the gastroenterologist needs to know. Frontline gastroenterology. 2011;2:96-104.
103. von Wagner C, Smith S, Halligan S, Ghanouni A, Power E, Lilford RJ, et al. Patient acceptability of CT colonography compared with double contrast barium enema: results from a multicentre randomised controlled trial of symptomatic patients. Eur Radiol. 2011;21(10):2046-55.
104. Koo BC, Ng CS, J UK-I, Prevost AT, Freeman AH. Minimal preparation CT for the diagnosis of suspected colorectal cancer in the frail and elderly patient. Clin Radiol. 2006;61(2):127-39.

105. Slater A, North M, Hart M, Ferrett C. Gas insufflation of minimal preparation CT of the colon reduces false-positives. Br J Radiol. 2012;85(1012):346-50.
106. Filippone A, Ambrosini R, Fuschi M, Marinelli T, Genovesi D, Bonomo L. Preoperative T and N staging of colorectal cancer: accuracy of contrast-enhanced multi-detector row CT colonography--initial experience. Radiology. 2004;231(1):83-90.
107. Group MS. Extramural depth of tumor invasion at thin-section MR in patients with rectal cancer: results of the MERCURY study. Radiology. 2007;243(1):132-9.
108. Taylor FG, Quirke P, Heald RJ, Moran B, Blomqvist L, Swift I, et al. One millimetre is the safe cut-off for magnetic resonance imaging prediction of surgical margin status in rectal cancer. Br J Surg. 2011;98(6):872-9.
109. Patel UB, Taylor F, Blomqvist L, George C, Evans H, Tekkis P, et al. Magnetic resonance imaging-detected tumor response for locally advanced rectal cancer predicts survival outcomes: MERCURY experience. J Clin Oncol. 2011;29(28):3753-60.
110. Puli SR, Reddy JB, Bechtold ML, Choudhary A, Antillon MR, Brugge WR. Accuracy of endoscopic ultrasound to diagnose nodal invasion by rectal cancers: a meta-analysis and systematic review. Ann Surg Oncol. 2009;16(5):1255-65.
111. Puli SR, Bechtold ML, Reddy JB, Choudhary A, Antillon MR, Brugge WR. How good is endoscopic ultrasound in differentiating various T stages of rectal cancer? Meta-analysis and systematic review. Ann Surg Oncol. 2009;16(2):254-65.
112. Puli SR, Bechtold ML, Reddy JB, Choudhary A, Antillon MR. Can endoscopic ultrasound predict early rectal cancers that can be resected endoscopically? A meta-analysis and systematic review. Dig Dis Sci. 2010;55(5):1221-9.
113. Briggs RH, Chowdhury FU, Lodge JP, Scarsbrook AF. Clinical impact of FDG PET-CT in patients with potentially operable metastatic colorectal cancer. Clin Radiol. 2011;66(12):1167-74.
114. MacDermid E, Hooton G, MacDonald M, McKay G, Grose D, Mohammed N, et al. Improving patient survival with the colorectal cancer multi-disciplinary team. Colorectal Dis. 2009;11(3):291-5.
115. Palmer G, Martling A, Cedermark B, Holm T. Preoperative tumour staging with multidisciplinary team assessment improves the outcome in locally advanced primary rectal cancer. Colorectal Dis. 2011;13(12):1361-9.
116. Tiernan J, Briggs CD, Irving GR, Swinscoe MT, Peterson M, Cameron IC. Evaluation of the introduction of a standardised protocol for the staging and follow-up of colorectal cancer on resection rates for liver metastases. Ann R Coll Surg Engl. 2010;92(3):225-30.

117. Heald RJ, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? Br J Surg. 1982;69(10):613-6.
118. den Dulk M, van de Velde CJ. Quality assurance in surgical oncology: the tale of the Dutch rectal cancer TME trial. J Surg Oncol. 2008;97(1):5-7.
119. Kapiteijn E, Putter H, van de Velde CJ, Cooperative investigators of the Dutch ColoRectal Cancer G. Impact of the introduction and training of total mesorectal excision on recurrence and survival in rectal cancer in The Netherlands. Br J Surg. 2002;89(9):1142-9.
120. Wibe A, Moller B, Norstein J, Carlsen E, Wiig JN, Heald RJ, et al. A national strategic change in treatment policy for rectal cancer--implementation of total mesorectal excision as routine treatment in Norway. A national audit. Dis Colon Rectum. 2002;45(7):857-66.
121. Martling A, Holm T, Rutqvist LE, Johansson H, Moran BJ, Heald RJ, et al. Impact of a surgical training programme on rectal cancer outcomes in Stockholm. Br J Surg. 2005;92(2):225-9.
122. Nagtegaal ID, van de Velde CJ, van der Worp E, Kapiteijn E, Quirke P, van Krieken JH, et al. Macroscopic evaluation of rectal cancer resection specimen: clinical significance of the pathologist in quality control. J Clin Oncol. 2002;20(7):1729-34.
123. Marr R, Birbeck K, Garvican J, Macklin CP, Tiffin NJ, Parsons WJ, et al. The modern abdominoperineal excision: the next challenge after total mesorectal excision. Ann Surg. 2005;242(1):74-82.
124. West NP, Finan PJ, Anderin C, Lindholm J, Holm T, Quirke P. Evidence of the oncologic superiority of cylindrical abdominoperineal excision for low rectal cancer. J Clin Oncol. 2008;26(21):3517-22.
125. Martijnse IS, Dudink RL, West NP, Wasowicz D, Nieuwenhuijzen GA, van Lijnschoten I, et al. Focus on extralevator perineal dissection in supine position for low rectal cancer has led to better quality of surgery and oncologic outcome. Ann Surg Oncol. 2012;19(3):786-93.
126. Lacy AM, Garcia-Valdecasas JC, Delgado S, Castells A, Taura P, Pique JM, et al. Laparoscopy-assisted colectomy versus open colectomy for treatment of non-metastatic colon cancer: a randomised trial. Lancet. 2002;359(9325):2224-9.
127. Veldkamp R, Kuhry E, Hop WC, Jeekel J, Kazemier G, Bonjer HJ, et al. Laparoscopic surgery versus open surgery for colon cancer: short-term outcomes of a randomised trial. Lancet Oncol. 2005;6(7):477-84.
128. Guillou PJ, Quirke P, Thorpe H, Walker J, Jayne DG, Smith AM, et al. Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal

cancer (MRC CLASICC trial): multicentre, randomised controlled trial. Lancet. 2005;365(9472):1718-26.
129. Berends FJ, Kazemier G, Bonjer HJ, Lange JF. Subcutaneous metastases after laparoscopic colectomy. Lancet. 1994;344(8914):58.
130. Clinical Outcomes of Surgical Therapy Study G, Nelson H, Sargent DJ, Wieand HS, Fleshman J, Anvari M, et al. A comparison of laparoscopically assisted and open colectomy for colon cancer. N Engl J Med. 2004;350(20):2050-9.
131. Colon Cancer Laparoscopic or Open Resection Study G, Buunen M, Veldkamp R, Hop WC, Kuhry E, Jeekel J, et al. Survival after laparoscopic surgery versus open surgery for colon cancer: long-term outcome of a randomised clinical trial. Lancet Oncol. 2009;10(1):44-52.
132. Green BL, Marshall HC, Collinson F, Quirke P, Guillou P, Jayne DG, et al. Long-term follow-up of the Medical Research Council CLASICC trial of conventional versus laparoscopically assisted resection in colorectal cancer. Br J Surg. 2013;100(1):75-82.
133. Jeong SY, Park JW, Nam BH, Kim S, Kang SB, Lim SB, et al. Open versus laparoscopic surgery for mid-rectal or low-rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial): survival outcomes of an open-label, non-inferiority, randomised controlled trial. Lancet Oncol. 2014;15(7):767-74.
134. Bonjer HJ, Deijen CL, Abis GA, Cuesta MA, van der Pas MH, de Lange-de Klerk ES, et al. A randomized trial of laparoscopic versus open surgery for rectal cancer. N Engl J Med. 2015;372(14):1324-32.
135. Fleshman J, Branda M, Sargent DJ, Boller AM, George V, Abbas M, et al. Effect of Laparoscopic-Assisted Resection vs Open Resection of Stage II or III Rectal Cancer on Pathologic Outcomes: The ACOSOG Z6051 Randomized Clinical Trial. JAMA. 2015;314(13):1346-55.
136. Stevenson AR, Solomon MJ, Lumley JW, Hewett P, Clouston AD, Gebski VJ, et al. Effect of Laparoscopic-Assisted Resection vs Open Resection on Pathological Outcomes in Rectal Cancer: The ALaCaRT Randomized Clinical Trial. JAMA. 2015;314(13):1356-63.
137. Collinson FJ, Jayne DG, Pigazzi A, Tsang C, Barrie JM, Edlin R, et al. An international, multicentre, prospective, randomised, controlled, unblinded, parallel-group trial of robotic-assisted versus standard laparoscopic surgery for the curative treatment of rectal cancer. Int J Colorectal Dis. 2012;27(2):233-41.
138. Colorectal Cancer Collaborative G. Adjuvant radiotherapy for rectal cancer: a systematic overview of 8,507 patients from 22 randomised trials. Lancet. 2001;358(9290):1291-304.

139. Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med. 2001;345(9):638-46.
140. Sauer R, Becker H, Hohenberger W, Rodel C, Wittekind C, Fietkau R, et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N Engl J Med. 2004;351(17):1731-40.
141. Ceelen WP, Van Nieuwenhove Y, Fierens K. Preoperative chemoradiation versus radiation alone for stage II and III resectable rectal cancer. Cochrane Database Syst Rev. 2009(1):CD006041.
142. Wolmark N, Rockette H, Fisher B, Wickerham DL, Redmond C, Fisher ER, et al. The benefit of leucovorin-modulated fluorouracil as postoperative adjuvant therapy for primary colon cancer: results from National Surgical Adjuvant Breast and Bowel Project protocol C-03. J Clin Oncol. 1993;11(10):1879-87.
143. Gill S, Loprinzi CL, Sargent DJ, Thome SD, Alberts SR, Haller DG, et al. Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: who benefits and by how much? J Clin Oncol. 2004;22(10):1797-806.
144. Wilkinson NW, Yothers G, Lopa S, Costantino JP, Petrelli NJ, Wolmark N. Long-term survival results of surgery alone versus surgery plus 5-fluorouracil and leucovorin for stage II and stage III colon cancer: pooled analysis of NSABP C-01 through C-05. A baseline from which to compare modern adjuvant trials. Ann Surg Oncol. 2010;17(4):959-66.
145. Twelves C, Wong A, Nowacki MP, Abt M, Burris H, 3rd, Carrato A, et al. Capecitabine as adjuvant treatment for stage III colon cancer. N Engl J Med. 2005;352(26):2696-704.
146. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med. 2004;350(23):2343-51.
147. Andre T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J Clin Oncol. 2009;27(19):3109-16.
148. Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators. J Clin Oncol. 1999;17(5):1356-63.
149. Quasar Collaborative G, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, et al. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet. 2007;370(9604):2020-9.

150. Benson AB, 3rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol. 2004;22(16):3408-19.
151. Guastadisegni C, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. Eur J Cancer. 2010;46(15):2788-98.
152. McArdle CS, Hole DJ. Outcome following surgery for colorectal cancer. Br Med Bull. 2002;64:119-25.
153. McArdle CS, Hole DJ. Outcome following surgery for colorectal cancer: analysis by hospital after adjustment for case-mix and deprivation. Br J Cancer. 2002;86(3):331-5.
154. Dukes CE. The classification of cancer of the rectum. J Pathol. 1932;35:323-32.
155. Turnbull RB, Jr., Kyle K, Watson FR, Spratt J. Cancer of the colon: the influence of the no-touch isolation technic on survival rates. Ann Surg. 1967;166(3):420-7.
156. Dukes CE, Bussey HJ. The spread of rectal cancer and its effect on prognosis. Br J Cancer. 1958;12(3):309-20.
157. Denoix P. Enquete permanent dans les centres anticancereaux. Bull Inst Natl Hyg. 1946;1:70-5.
158. Sobin LH, Hermanek P, Hutter RV. TNM classification of malignant tumors. A comparison between the new (1987) and the old editions. Cancer. 1988;61(11):2310-4.
159. TNM Classification of Malignant Tumours, 7th Edition: Wiley-Blackwell; 2009. 336 p.
160. Quirke P, Williams GT, Ectors N, Ensari A, Piard F, Nagtegaal I. The future of the TNM staging system in colorectal cancer: time for a debate? Lancet Oncol. 2007;8(7):651-7.
161. Morris EJ, Maughan NJ, Forman D, Quirke P. Identifying stage III colorectal cancer patients: the influence of the patient, surgeon, and pathologist. J Clin Oncol. 2007;25(18):2573-9.
162. O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst. 2004;96(19):1420-5.
163. Morris EJ, Maughan NJ, Forman D, Quirke P. Who to treat with adjuvant therapy in Dukes B/stage II colorectal cancer? The need for high quality pathology. Gut. 2007;56(10):1419-25.
164. Network NCI. Colorectal Cancer Survival by Stage - NCIN Data Briefing: National Cancer Intelligence Network; 2010 [updated June 2009.

165. Kang H, O'Connell JB, Maggard MA, Sack J, Ko CY. A 10-year outcomes evaluation of mucinous and signet-ring cell carcinoma of the colon and rectum. Dis Colon Rectum. 2005;48(6):1161-8.
166. Nagtegaal I, Gaspar C, Marijnen C, Van De Velde C, Fodde R, Van Krieken H. Morphological changes in tumour type after radiotherapy are accompanied by changes in gene expression profile but not in clinical behaviour. J Pathol. 2004;204(2):183-92.
167. Verhulst J, Ferdinande L, Demetter P, Ceelen W. Mucinous subtype as prognostic factor in colorectal cancer: a systematic review and meta-analysis. J Clin Pathol. 2012;65(5):381-8.
168. Compton CC. Updated protocol for the examination of specimens from patients with carcinomas of the colon and rectum, excluding carcinoid tumors, lymphomas, sarcomas, and tumors of the vermiform appendix: a basis for checklists. Cancer Committee. Arch Pathol Lab Med. 2000;124(7):1016-25.
169. Jass JR, Atkin WS, Cuzick J, Bussey HJ, Morson BC, Northover JM, et al. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. Histopathology. 1986;10(5):437-59.
170. Halvorsen TB, Seim E. Degree of differentiation in colorectal adenocarcinomas: a multivariate analysis of the influence on survival. J Clin Pathol. 1988;41(5):532-7.
171. Shepherd NA, Baxter KJ, Love SB. The prognostic importance of peritoneal involvement in colonic cancer: a prospective evaluation. Gastroenterology. 1997;112(4):1096-102.
172. Petersen VC, Baxter KJ, Love SB, Shepherd NA. Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer. Gut. 2002;51(1):65-9.
173. Porter GA, O'Keefe GE, Yakimets WW. Inadvertent perforation of the rectum during abdominoperineal resection. Am J Surg. 1996;172(4):324-7.
174. Quirke P, Durdey P, Dixon MF, Williams NS. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histopathological study of lateral tumour spread and surgical excision. Lancet. 1986;2(8514):996-9.
175. Adam IJ, Mohamdee MO, Martin IG, Scott N, Finan PJ, Johnston D, et al. Role of circumferential margin involvement in the local recurrence of rectal cancer. Lancet. 1994;344(8924):707-11.
176. Wittekind C, Compton C, Quirke P, Nagtegaal I, Merkel S, Hermanek P, et al. A uniform residual tumor (R) classification: integration of the R classification and the circumferential margin status. Cancer. 2009;115(15):3483-8.

177. Quirke P, Steele R, Monson J, Grieve R, Khanna S, Couture J, et al. Effect of the plane of surgery achieved on local recurrence in patients with operable rectal cancer: a prospective study using data from the MRC CR07 and NCIC-CTG CO16 randomised clinical trial. Lancet. 2009;373(9666):821-8.
178. Garcia-Granero E, Faiz O, Munoz E, Flor B, Navarro S, Faus C, et al. Macroscopic assessment of mesorectal excision in rectal cancer: a useful tool for improving quality control in a multidisciplinary team. Cancer. 2009;115(15):3400-11.
179. Loughrey MB, Quirke P, Shepherd NA. Dataset for colorectal cancer histopathology reports. 3 ed: The Royal College of Pathologists; 2014 July 2014.
180. Chang GJ, Rodriguez-Bigas MA, Skibber JM, Moyer VA. Lymph node evaluation and survival after curative resection of colon cancer: systematic review. J Natl Cancer Inst. 2007;99(6):433-41.
181. Ceelen W, Van Nieuwenhove Y, Pattyn P. Prognostic value of the lymph node ratio in stage III colorectal cancer: a systematic review. Ann Surg Oncol. 2010;17(11):2847-55.
182. Tong LL, Gao P, Wang ZN, Song YX, Xu YY, Sun Z, et al. Can lymph node ratio take the place of pN categories in the UICC/AJCC TNM classification system for colorectal cancer? Ann Surg Oncol. 2011;18(9):2453-60.
183. Lee HY, Choi HJ, Park KJ, Shin JS, Kwon HC, Roh MS, et al. Prognostic significance of metastatic lymph node ratio in node-positive colon carcinoma. Ann Surg Oncol. 2007;14(5):1712-7.
184. Park IJ, Choi GS, Jun SH. Nodal stage of stage III colon cancer: the impact of metastatic lymph node ratio. J Surg Oncol. 2009;100(3):240-3.
185. Vaccaro CA, Im V, Rossi GL, Quintana GO, Benati ML, Perez de Arenaza D, et al. Lymph node ratio as prognosis factor for colon cancer treated by colorectal surgeons. Dis Colon Rectum. 2009;52(7):1244-50.
186. Talbot IC, Ritchie S, Leighton M, Hughes AO, Bussey HJ, Morson BC. Invasion of veins by carcinoma of rectum: method of detection, histological features and significance. Histopathology. 1981;5(2):141-63.
187. Talbot IC, Ritchie S, Leighton MH, Hughes AO, Bussey HJ, Morson BC. The clinical significance of invasion of veins by rectal cancer. Br J Surg. 1980;67(6):439-42.
188. Roxburgh CS, McMillan DC, Anderson JH, McKee RF, Horgan PG, Foulis AK. Elastica staining for venous invasion results in superior prediction of cancer-specific survival in colorectal cancer. Ann Surg. 2010;252(6):989-97.
189. Betge J, Pollheimer MJ, Lindtner RA, Kornprat P, Schlemmer A, Rehak P, et al. Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. Cancer. 2012;118(3):628-38.

190. Messenger DE, Driman DK, McLeod RS, Riddell RH, Kirsch R. Current practice patterns among pathologists in the assessment of venous invasion in colorectal cancer. J Clin Pathol. 2011;64(11):983-9.
191. Messenger DE, Driman DK, Kirsch R. Developments in the assessment of venous invasion in colorectal cancer: implications for future practice and patient outcome. Hum Pathol. 2012;43(7):965-73.
192. Batsakis JG. Nerves and neurotropic carcinomas. Ann Otol Rhinol Laryngol. 1985;94(4 Pt 1):426-7.
193. Ueno H, Hase K, Mochizuki H. Criteria for extramural perineural invasion as a prognostic factor in rectal cancer. Br J Surg. 2001;88(7):994-1000.
194. Weiser MR, Landmann RG, Kattan MW, Gonen M, Shia J, Chou J, et al. Individualized prediction of colon cancer recurrence using a nomogram. J Clin Oncol. 2008;26(3):380-5.
195. Silberfein EJ, Kattepogu KM, Hu CY, Skibber JM, Rodriguez-Bigas MA, Feig B, et al. Long-term survival and recurrence outcomes following surgery for distal rectal cancer. Ann Surg Oncol. 2010;17(11):2863-9.
196. Huh JW, Kim HR, Kim YJ. Lymphovascular or perineural invasion may predict lymph node metastasis in patients with T1 and T2 colorectal cancer. J Gastrointest Surg. 2010;14(7):1074-80.
197. Liebig C, Ayala G, Wilks J, Verstovsek G, Liu H, Agarwal N, et al. Perineural invasion is an independent predictor of outcome in colorectal cancer. J Clin Oncol. 2009;27(31):5131-7.
198. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol. 2005;23(3):609-18.
199. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med. 2003;349(3):247-57.
200. Deschoolmeester V, Baay M, Lardon F, Pauwels P, Peeters M. Immune Cells in Colorectal Cancer: Prognostic Relevance and Role of MSI. Cancer Microenviron. 2011;4(3):377-92.
201. Ogino S, Nosho K, Irahara N, Meyerhardt JA, Baba Y, Shima K, et al. Lymphocytic reaction to colorectal cancer is associated with longer survival, independent of lymph node count, microsatellite instability, and CpG island methylator phenotype. Clin Cancer Res. 2009;15(20):6412-20.
202. Munro AJ, Lain S, Lane DP. P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer. 2005;92(3):434-44.

203. Kelley RK, Venook AP. Prognostic and predictive markers in stage II colon cancer: is there a role for gene expression profiling? Clin Colorectal Cancer. 2011;10(2):73-80.
204. Salazar R, Roepman P, Capella G, Moreno V, Simon I, Dreezen C, et al. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. J Clin Oncol. 2011;29(1):17-24.
205. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010;11(8):753-62.
206. Hutchins G, Southward K, Handley K, Magill L, Beaumont C, Stahlschmidt J, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol. 2011;29(10):1261-70.
207. Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol. 2010;28(3):466-74.
208. Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. N Engl J Med. 2012;367(17):1596-606.
209. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357(9255):539-45.
210. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
211. Ferrone C, Dranoff G. Dual roles for immunity in gastrointestinal cancers. J Clin Oncol. 2010;28(26):4045-51.
212. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.
213. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315(26):1650-9.
214. Kabelitz D, Medzhitov R. Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines. Curr Opin Immunol. 2007;19(1):1-3.
215. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140(6):883-99.
216. Bollrath J, Greten FR. IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. EMBO Rep. 2009;10(12):1314-9.

217. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 2010;21(1):11-9.
218. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009;9(11):798-809.
219. Jain S, Harris J, Ware J. Platelets: linking hemostasis and cancer. Arterioscler Thromb Vasc Biol. 2010;30(12):2362-7.
220. Verbeke H, Struyf S, Laureys G, Van Damme J. The expression and role of CXC chemokines in colorectal cancer. Cytokine Growth Factor Rev. 2011;22(5-6):345-58.
221. Sarvaiya PJ, Guo D, Ulasov I, Gabikian P, Lesniak MS. Chemokines in tumor progression and metastasis. Oncotarget. 2013;4(12):2171-85.
222. Van den Eynden GG, Majeed AW, Illemann M, Vermeulen PB, Bird NC, Hoyer-Hansen G, et al. The multifaceted role of the microenvironment in liver metastasis: biology and clinical implications. Cancer Res. 2013;73(7):2031-43.
223. Kim J, Takeuchi H, Lam ST, Turner RR, Wang HJ, Kuo C, et al. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. J Clin Oncol. 2005;23(12):2744-53.
224. Homey B, Muller A, Zlotnik A. Chemokines: agents for the immunotherapy of cancer? Nat Rev Immunol. 2002;2(3):175-84.
225. Roxburgh CSD, McMillan DC. The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treat Rev. 2012;38(5):451-66.
226. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121(1):1-14.
227. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med. 2005;353(25):2654-66.
228. Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. Genes Dev. 2007;21(11):1396-408.
229. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007;7(1):41-51.
230. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity. 1999;10(1):39-49.

231. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med. 2005;11(12):1314-21.
232. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960-4.
233. Richards CH, Flegg KM, Roxburgh CSD, Going JJ, Mohammed Z, Horgan PG, et al. The relationships between cellular components of the peritumoural inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer. Br J Cancer. 2012;106(12):2010-5.
234. Morris M, Platell C, Iacopetta B. Tumor-infiltrating lymphocytes and perforation in colon cancer predict positive response to 5-fluorouracil chemotherapy. Clin Cancer Res. 2008;14(5):1413-7.
235. Richards CH, Roxburgh CS, Anderson JH, McKee RF, Foulis AK, Horgan PG, et al. Prognostic value of tumour necrosis and host inflammatory responses in colorectal cancer. Br J Surg. 2012;99(2):287-94.
236. Lieubeau B, Heymann MF, Henry F, Barbieux I, Meflah K, Gregoire M. Immunomodulatory effects of tumor-associated fibroblasts in colorectal-tumor development. Int J Cancer. 1999;81(4):629-36.
237. Ueno H, Jones AM, Wilkinson KH, Jass JR, Talbot IC. Histological categorisation of fibrotic cancer stroma in advanced rectal cancer. Gut. 2004;53(4):581-6.
238. Berghoff AS, Fuchs E, Ricken G, Mlecnik B, Bindea G, Spanberger T, et al. Density of tumor-infiltrating lymphocytes correlates with extent of brain edema and overall survival time in patients with brain metastases. Oncoimmunology. 2016;5(1):e1057388.
239. Jass JR. Lymphocytic infiltration and survival in rectal cancer. J Clin Pathol. 1986;39(6):585-9.
240. Jass JR, Love SB, Northover JM. A new prognostic classification of rectal cancer. Lancet. 1987;1(8545):1303-6.
241. Fisher ER, Robinsky B, Sass R, Fisher B. Relative prognostic value of the Dukes and the Jass systems in rectal cancer. Findings from the National Surgical Adjuvant Breast and Bowel Projects (Protocol R-01). Dis Colon Rectum. 1989;32(11):944-9.
242. Deans GT, Heatley M, Anderson N, Patterson CC, Rowlands BJ, Parks TG, et al. Jass' classification revisited. J Am Coll Surg. 1994;179(1):11-7.
243. Roxburgh CS, Salmond JM, Horgan PG, Oien KA, McMillan DC. Comparison of the prognostic value of inflammation-based pathologic and biochemical criteria in patients

undergoing potentially curative resection for colorectal cancer. Ann Surg. 2009;249(5):788-93.
244. Jass JR, Ajioka Y, Allen JP, Chan YF, Cohen RJ, Nixon JM, et al. Assessment of invasive growth pattern and lymphocytic infiltration in colorectal cancer. Histopathology. 1996;28(6):543-8.
245. Graham DM, Appelman HD. Crohn's-like lymphoid reaction and colorectal carcinoma: a potential histologic prognosticator. Mod Pathol. 1990;3(3):332-5.
246. Kakar S, Aksoy S, Burgart LJ, Smyrk TC. Mucinous carcinoma of the colon: correlation of loss of mismatch repair enzymes with clinicopathologic features and survival. Mod Pathol. 2004;17(6):696-700.
247. Jenkins MA, Hayashi S, O'Shea AM, Burgart LJ, Smyrk TC, Shimizu D, et al. Pathology features in Bethesda guidelines predict colorectal cancer microsatellite instability: a population-based study. Gastroenterology. 2007;133(1):48-56.
248. Klintrup K, Makinen JM, Kauppila S, Vare PO, Melkko J, Tuominen H, et al. Inflammation and prognosis in colorectal cancer. Eur J Cancer. 2005;41(17):2645-54.
249. Roxburgh C, Salmond J, Horgan P, Oien K, McMillan D. Tumour inflammatory infiltrate predicts survival following curative resection for node-negative colorectal cancer. Eur J Cancer. 2009;45(12):2138-45.
250. Dutta S, Going JJ, Crumley AB, Mohammed Z, Orange C, Edwards J, et al. The relationship between tumour necrosis, tumour proliferation, local and systemic inflammation, microvessel density and survival in patients undergoing potentially curative resection of oesophageal adenocarcinoma. Br J Cancer. 2012;106(4):702-10.
251. Mohammed ZM, Going JJ, Edwards J, Elsberger B, Doughty JC, McMillan DC. The relationship between components of tumour inflammatory cell infiltrate and clinicopathological factors and survival in patients with primary operable invasive ductal breast cancer. Br J Cancer. 2012;107(5):864-73.
252. Ueno H, Hashiguchi Y, Shimazaki H, Shinto E, Kajiwara Y, Nakanishi K, et al. Objective criteria for crohn-like lymphoid reaction in colorectal cancer. Am J Clin Pathol. 2013;139(4):434-41.
253. Nagtegaal ID, Marijnen CA, Kranenbarg EK, Mulder-Stapel A, Hermans J, van de Velde CJ, et al. Local and distant recurrences in rectal cancer patients are predicted by the nonspecific immune response; specific immune response has only a systemic effect--a histopathological and immunohistochemical study. BMC cancer. 2001;1:7.
254. Deschoolmeester V, Baay M, Van Marck E, Weyler J, Vermeulen P, Lardon F, et al. Tumor infiltrating lymphocytes: an intriguing player in the survival of colorectal cancer patients. BMC Immunol. 2010;11(1):19.

255. Lugli A, Karamitopoulou E, Panayiotides I, Karakitsos P, Rallis G, Peros G, et al. CD8+ lymphocytes/ tumour-budding index: an independent prognostic factor representing a 'pro-/anti-tumour' approach to tumour host interaction in colorectal cancer. Br J Cancer. 2009;101(8):1382-92.
256. Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. J Pathol. 2010;222(4):350-66.
257. Galon J, Pages F, Marincola FM, Angell HK, Thurin M, Lugli A, et al. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med. 2012;10:205.
258. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298-306.
259. Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol. 2009;27(35):5944-51.
260. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol. 2011;29(6):610-8.
261. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340(6):448-54.
262. Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol. 2002;2(10):787-95.
263. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436-44.
264. Shay JE, Celeste Simon M. Hypoxia-inducible factors: crosstalk between inflammation and metabolism. Semin Cell Dev Biol. 2012;23(4):389-94.
265. McMillan DC, Scott HR, Watson WS, Preston T, Milroy R, McArdle CS. Longitudinal study of body cell mass depletion and the inflammatory response in cancer patients. Nutr Cancer. 1998;31(2):101-5.
266. McMillan DC, Wigmore SJ, Fearon KC, O'Gorman P, Wright CE, McArdle CS. A prospective randomized study of megestrol acetate and ibuprofen in gastrointestinal cancer patients with weight loss. Br J Cancer. 1999;79(3-4):495-500.
267. Forrest LM, McMillan DC, McArdle CS, Angerson WJ, Dunlop DJ. Comparison of an inflammation-based prognostic score (GPS) with performance status (ECOG) in patients receiving platinum-based chemotherapy for inoperable non-small-cell lung cancer. Br J Cancer. 2004;90(9):1704-6.

268. Fearon K, Strasser F, Anker SD, Bosaeus I, Bruera E, Fainsinger RL, et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol. 2011;12(5):489-95.
269. McMillan DC, Crozier JE, Canna K, Angerson WJ, McArdle CS. Evaluation of an inflammation-based prognostic score (GPS) in patients undergoing resection for colon and rectal cancer. Int J Colorectal Dis. 2007;22(8):881-6.
270. Moyes LH, Leitch EF, McKee RF, Anderson JH, Horgan PG, McMillan DC. Preoperative systemic inflammation predicts postoperative infectious complications in patients undergoing curative resection for colorectal cancer. Br J Cancer. 2009;100(8):1236-9.
271. Clarke SJ, Chua W, Moore M, Kao S, Phan V, Tan C, et al. Use of inflammatory markers to guide cancer treatment. Clin Pharmacol Ther. 2011;90(3):475-8.
272. Castell JV, Gomez-Lechon MJ, David M, Fabra R, Trullenque R, Heinrich PC. Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. Hepatology. 1990;12(5):1179-86.
273. Hogarth MB, Gallimore R, Savage P, Palmer AJ, Starr JM, Bulpitt CJ, et al. Acute phase proteins, C-reactive protein and serum amyloid A protein, as prognostic markers in the elderly inpatient. Age Ageing. 1997;26(2):153-8.
274. Walsh SR, Cook EJ, Goulder F, Justin TA, Keeling NJ. Neutrophil-lymphocyte ratio as a prognostic factor in colorectal cancer. J Surg Oncol. 2005;91(3):181-4.
275. Smith RA, Bosonnet L, Raraty M, Sutton R, Neoptolemos JP, Campbell F, et al. Preoperative platelet-lymphocyte ratio is an independent significant prognostic marker in resected pancreatic ductal adenocarcinoma. Am J Surg. 2009;197(4):466-72.
276. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann Intern Med. 1994;121(4):241-6.
277. Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, et al. Aspirin and the risk of colorectal cancer in women. N Engl J Med. 1995;333(10):609-14.
278. Benedetti AL, Collet JP, Boivin JF, Hanley JA. Effect of nonsteroidal anti-inflammatory drugs on stage of colon cancer at diagnosis. J Clin Epidemiol. 2003;56(8):782-7.
279. Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. Lancet. 2012;379(9826):1591-601.

280. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet. 2007;369(9573):1603-13.
281. Chia WK, Ali R, Toh HC. Aspirin as adjuvant therapy for colorectal cancer-reinterpreting paradigms. Nat Rev Clin Oncol. 2012;9(10):561-70.
282. Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW, Jr. Aspirin use and risk of fatal cancer. Cancer Res. 1993;53(6):1322-7.
283. Thun MJM, Namboodiri MMM, Heath CWC. Aspirin use and reduced risk of fatal colon cancer. The New England journal of medicine. 1991;325(23):1593-6.
284. Zell JA, Ziogas A, Bernstein L, Clarke CA, Deapen D, Largent JA, et al. Nonsteroidal anti-inflammatory drugs: effects on mortality after colorectal cancer diagnosis. Cancer. 2009;115(24):5662-71.
285. Coghill AE, Newcomb PA, Campbell PT, Burnett-Hartman AN, Adams SV, Poole EM, et al. Prediagnostic non-steroidal anti-inflammatory drug use and survival after diagnosis of colorectal cancer. Gut. 2011;60(4):491-8.
286. Lynch D, Murphy A. The emerging role of immunotherapy in colorectal cancer. Ann Transl Med. 2016;4(16):305.
287. Bilusic M, Heery CR, Arlen PM, Rauckhorst M, Apelian D, Tsang KY, et al. Phase I trial of a recombinant yeast-CEA vaccine (GI-6207) in adults with metastatic CEA-expressing carcinoma. Cancer Immunol Immunother. 2014;63(3):225-34.
288. Vermorken JB, Claessen AM, van Tinteren H, Gall HE, Ezinga R, Meijer S, et al. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. Lancet. 1999;353(9150):345-50.
289. Harris JE, Ryan L, Hoover HC, Jr., Stuart RK, Oken MM, Benson AB, 3rd, et al. Adjuvant active specific immunotherapy for stage II and III colon cancer with an autologous tumor cell vaccine: Eastern Cooperative Oncology Group Study E5283. J Clin Oncol. 2000;18(1):148-57.
290. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12(4):269-81.
291. Zhen YH, Liu XH, Yang Y, Li B, Tang JL, Zeng QX, et al. Phase I/II study of adjuvant immunotherapy with sentinel lymph node T lymphocytes in patients with colorectal cancer. Cancer Immunol Immunother. 2015;64(9):1083-93.
292. Nirschl CJ, Drake CG. Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. Clin Cancer Res. 2013;19(18):4917-24.

293. Chung KY, Gore I, Fong L, Venook A, Beck SB, Dorazio P, et al. Phase II study of the anti-cytotoxic T-lymphocyte-associated antigen 4 monoclonal antibody, tremelimumab, in patients with refractory metastatic colorectal cancer. J Clin Oncol. 2010;28(21):3485-90.
294. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015;372(26):2509-20.
295. Llosa NJ, Cruise M, Tam A, Wicks EC, Hechenbleikner EM, Taube JM, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov. 2015;5(1):43-51.
296. Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. JAMA. 2009;302(6):649-58.
297. Bastiaannet E, Sampieri K, Dekkers OM, de Craen AJ, van Herk-Sukel MP, Lemmens V, et al. Use of aspirin postdiagnosis improves survival for colon cancer patients. Br J Cancer. 2012;106(9):1564-70.
298. Fuchs CS, Meyerhardt JA, Heseltine DL, Niedzwiecki D, Hollis D, Chan AT, et al. Influence of regular aspirin use on survival for patients with stage III colon cancer: Findings from Intergroup trial CALGB 89803. J Clin Oncol. 2005;23(16S):3530.
299. Midgley RS, McConkey CC, Johnstone EC, Dunn JA, Smith JL, Grumett SA, et al. Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. J Clin Oncol. 2010;28(30):4575-80.
300. Phase III Randomized Study of Adjuvant Celecoxib With Fluorouracil and Leucovorin Calcium in Patients With Curatively Resected Stage III Adenocarcinoma of the Colon National Cancer Institute: National Institutes of Health; 2004 [updated 22/6/2006. Available from: http://cancer.gov/clinicaltrials/search/view?cdrid=367335&version=healthprofessional.
301. Davis TW, Hunter N, Trifan OC, Milas L, Masferrer JL. COX-2 inhibitors as radiosensitizing agents for cancer therapy. Am J Clin Oncol. 2003;26(4):S58-61.
302. Debucquoy A, Roels S, Goethals L, Libbrecht L, Van Cutsem E, Geboes K, et al. Double blind randomized phase II study with radiation+5-fluorouracil+/-celecoxib for resectable rectal cancer. Radiother Oncol. 2009;93(2):273-8.
303. Ali R, Toh HC, Chia WK, Investigators AT. The utility of Aspirin in Dukes C and High Risk Dukes B Colorectal cancer--the ASCOLT study: study protocol for a randomized controlled trial. Trials. 2011;12:261.
304. Church RD, Fleshman JW, McLeod HL. Cyclo-oxygenase 2 inhibition in colorectal cancer therapy. Br J Surg. 2003;90(9):1055-67.

305. Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. Annu Rev Med. 2007;58:239-52.
306. Fenwick SW, Toogood GJ, Lodge JP, Hull MA. The effect of the selective cyclooxygenase-2 inhibitor rofecoxib on human colorectal cancer liver metastases. Gastroenterology. 2003;125(3):716-29.
307. Chalmers CR, Wilson DJ, Ward J, Robinson PJ, Toogood GJ, Hull MA. Antiangiogenic activity of the selective cyclooxygenase 2 inhibitor rofecoxib in human colorectal cancer liver metastases. Gut. 2006;55(7):1058-9.
308. Auman JT, Church R, Lee SY, Watson MA, Fleshman JW, McLeod HL. Celecoxib pre-treatment in human colorectal adenocarcinoma patients is associated with gene expression alterations suggestive of diminished cellular proliferation. Eur J Cancer. 2008;44(12):1754-60.
309. Lonnroth C, Andersson M, Nordgren S, Lundholm K. Downregulation of Prominin 1/CD133 expression in colorectal cancer by NSAIDs following short-term preoperative treatment. Int J Oncol. 2012;41(1):15-23.
310. Tuynman JB, Peppelenbosch MP, Richel DJ. COX-2 inhibition as a tool to treat and prevent colorectal cancer. Crit Rev Oncol Hematol. 2004;52(2):81-101.
311. Schror K. Pharmacology and cellular/molecular mechanisms of action of aspirin and non-aspirin NSAIDs in colorectal cancer. Best Pract Res Clin Gastroenterol. 2011;25(4-5):473-84.
312. Dimberg J, Samuelsson A, Hugander A, Soderkvist P. Differential expression of cyclooxygenase 2 in human colorectal cancer. Gut. 1999;45(5):730-2.
313. Nasir A, Kaiser HE, Boulware D, Hakam A, Zhao H, Yeatman T, et al. Cyclooxygenase-2 expression in right- and left-sided colon cancer: a rationale for optimization of cyclooxygenase-2 inhibitor therapy. Clin Colorectal Cancer. 2004;3(4):243-7.
314. Gustafsson A, Hansson E, Kressner U, Nordgren S, Andersson M, Wang W, et al. EP1-4 subtype, COX and PPAR gamma receptor expression in colorectal cancer in prediction of disease-specific mortality. Int J Cancer. 2007;121(2):232-40.
315. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med. 2007;356(21):2131-42.
316. Barry EL, Sansbury LB, Grau MV, Ali IU, Tsang S, Munroe DJ, et al. Cyclooxygenase-2 polymorphisms, aspirin treatment, and risk for colorectal adenoma recurrence--data from a randomized clinical trial. Cancer Epidemiol Biomarkers Prev. 2009;18(10):2726-33.

317. Krishnan K, Ruffin MT, Normolle D, Shureiqi I, Burney K, Bailey J, et al. Colonic mucosal prostaglandin E2 and cyclooxygenase expression before and after low aspirin doses in subjects at high risk or at normal risk for colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2001;10(5):447-53.
318. Thun MJ, Jacobs EJ, Patrono C. The role of aspirin in cancer prevention. Nat Rev Clin Oncol. 2012;9(5):259-67.
319. Sciulli MG, Filabozzi P, Tacconelli S, Padovano R, Ricciotti E, Capone ML, et al. Platelet activation in patients with colorectal cancer. Prostaglandins Leukot Essent Fatty Acids. 2005;72(2):79-83.
320. Grosch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst. 2006;98(11):736-47.
321. Boon EM, Keller JJ, Wormhoudt TA, Giardiello FM, Offerhaus GJ, van der Neut R, et al. Sulindac targets nuclear beta-catenin accumulation and Wnt signalling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines. Br J Cancer. 2004;90(1):224-9.
322. Greenspan EJ, Madigan JP, Boardman LA, Rosenberg DW. Ibuprofen inhibits activation of nuclear {beta}-catenin in human colon adenomas and induces the phosphorylation of GSK-3{beta}. Cancer Prev Res (Phila). 2011;4(1):161-71.
323. Seufert BL, Poole EM, Whitton J, Xiao L, Makar KW, Campbell PT, et al. IkappaBKbeta and NFkappaB1, NSAID use and risk of colorectal cancer in the Colon Cancer Family Registry. Carcinogenesis. 2013;34(1):79-85.
324. Chan AT, Sima CS, Zauber AG, Ridker PM, Hawk ET, Bertagnolli MM. C-reactive protein and risk of colorectal adenoma according to celecoxib treatment. Cancer Prev Res (Phila). 2011;4(8):1172-80.
325. Hussain M, Javeed A, Ashraf M, Al-Zaubai N, Stewart A, Mukhtar MM. Non-steroidal anti-inflammatory drugs, tumour immunity and immunotherapy. Pharmacol Res. 2012;66(1):7-18.
326. Lonnroth C, Andersson M, Arvidsson A, Nordgren S, Brevinge H, Lagerstedt K, et al. Preoperative treatment with a non-steroidal anti-inflammatory drug (NSAID) increases tumor tissue infiltration of seemingly activated immune cells in colorectal cancer. Cancer Immun. 2008;8:5.
327. Yaqub S, Henjum K, Mahic M, Jahnsen FL, Aandahl EM, Bjornbeth BA, et al. Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner. Cancer Immunol Immunother. 2008;57(6):813-21.

328. Markewitz A, Faist E, Lang S, Endres S, Fuchs D, Reichart B. Successful restoration of cell-mediated immune response after cardiopulmonary bypass by immunomodulation. J Thorac Cardiovasc Surg. 1993;105(1):15-24.
329. Gogos CA, Maroulis J, Zoumbos NC, Salsa B, Kalfarentzos F. Effect of Parenteral Indomethacin on T-Lymphocyte Subpopulations and Cytokine Production in Patients under Major Surgical Operations. Res Exp Med (Berl). 1995;195(2):85-92.
330. Han T, Nemoto T, Ledesma EJ, Bruno S. Enhancement of T lymphocyte proliferative response to mitogens by indomethacin in breast and colorectal cancer patients. Int J Immunopharmacol. 1983;5(1):11-5.
331. Balch CM, Dougherty PA, Cloud GA, Tilden AB. Prostaglandin E2-mediated suppression of cellular immunity in colon cancer patients. Surgery. 1984;95(1):71-7.
332. Ho GY, Xue X, Cushman M, McKeown-Eyssen G, Sandler RS, Ahnen DJ, et al. Antagonistic effects of aspirin and folic acid on inflammation markers and subsequent risk of recurrent colorectal adenomas. J Natl Cancer Inst. 2009;101(23):1650-4.
333. Konturek PC, Rembiasz K, Burnat G, Konturek SJ, Tusinela M, Bielanski W, et al. Effects of cyclooxygenase-2 inhibition on serum and tumor gastrins and expression of apoptosis-related proteins in colorectal cancer. Dig Dis Sci. 2006;51(4):779-87.
334. McMillan DC, Leen E, Smith J, Sturgeon C, Preston T, Cooke TG, et al. Effect of extended ibuprofen administration on the acute phase protein response in colorectal cancer patients. Eur J Surg Oncol. 1995;21(5):531-4.
335. Coogan PF, Smith J, Rosenberg L. Statin use and risk of colorectal cancer. J Natl Cancer Inst. 2007;99(1):32-40.
336. Siddiqui AA, Nazario H, Mahgoub A, Patel M, Cipher D, Spechler SJ. For patients with colorectal cancer, the long-term use of statins is associated with better clinical outcomes. Dig Dis Sci. 2009;54(6):1307-11.
337. Ng K, Ogino S, Meyerhardt JA, Chan JA, Chan AT, Niedzwiecki D, et al. Relationship between statin use and colon cancer recurrence and survival: results from CALGB 89803. J Natl Cancer Inst. 2011;103(20):1540-51.
338. Lipkin SM, Chao EC, Moreno V, Rozek LS, Rennert H, Pinchev M, et al. Genetic variation in 3-hydroxy-3-methylglutaryl CoA reductase modifies the chemopreventive activity of statins for colorectal cancer. Cancer Prev Res (Phila). 2010;3(5):597-603.
339. Rosuvastatin in Treating Patients With Stage I or Stage II Colon Cancer That Was Removed By Surgery 2009 [updated 09/02/20125/12/2012]. Available from: http://clinicaltrials.gov/show/NCT01011478.

340. Agarwal B, Bhendwal S, Halmos B, Moss SF, Ramey WG, Holt PR. Lovastatin augments apoptosis induced by chemotherapeutic agents in colon cancer cells. Clin Cancer Res. 1999;5(8):2223-9.
341. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell. 2003;4(4):257-62.
342. Wachtershauser A, Akoglu B, Stein J. HMG-CoA reductase inhibitor mevastatin enhances the growth inhibitory effect of butyrate in the colorectal carcinoma cell line Caco-2. Carcinogenesis. 2001;22(7):1061-7.
343. Qi XF, Kim DH, Yoon YS, Kim SK, Cai DQ, Teng YC, et al. Involvement of oxidative stress in simvastatin-induced apoptosis of murine CT26 colon carcinoma cells. Toxicol Lett. 2010;199(3):277-87.
344. Skaletz-Rorowski A, Walsh K. Statin therapy and angiogenesis. Curr Opin Lipidol. 2003;14(6):599-603.
345. Hinoue T, Weisenberger DJ, Pan F, Campan M, Kim M, Young J, et al. Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. PloS one. 2009;4(12):e8357.
346. Kodach LL, Jacobs RJ, Voorneveld PW, Wildenberg ME, Verspaget HW, van Wezel T, et al. Statins augment the chemosensitivity of colorectal cancer cells inducing epigenetic reprogramming and reducing colorectal cancer cell 'stemness' via the bone morphogenetic protein pathway. Gut. 2011;60(11):1544-53.
347. Wang W, Collie-Duguid E, Cassidy J. Cerivastatin enhances the cytotoxicity of 5-fluorouracil on chemosensitive and resistant colorectal cancer cell lines. FEBS Lett. 2002;531(3):415-20.
348. Riganti C, Doublier S, Costamagna C, Aldieri E, Pescarmona G, Ghigo D, et al. Activation of nuclear factor-kappa B pathway by simvastatin and RhoA silencing increases doxorubicin cytotoxicity in human colon cancer HT29 cells. Mol Pharmacol. 2008;74(2):476-84.
349. Lee J, Lee I, Han B, Park JO, Jang J, Park C, et al. Effect of simvastatin on cetuximab resistance in human colorectal cancer with KRAS mutations. J Natl Cancer Inst. 2011;103(8):674-88.
350. Katz MS, Minsky BD, Saltz LB, Riedel E, Chessin DB, Guillem JG. Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys. 2005;62(5):1363-70.
351. Jain MK, Ridker PM. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. Nat Rev Drug Discov. 2005;4(12):977-87.

352. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. Nat Med. 2000;6(12):1399-402.
353. Samadder NJ, Mukherjee B, Huang SC, Ahn J, Rennert HS, Greenson JK, et al. Risk of colorectal cancer in self-reported inflammatory bowel disease and modification of risk by statin and NSAID use. Cancer. 2011;117(8):1640-8.
354. Lee JY, Kim JS, Kim JM, Kim N, Jung HC, Song IS. Simvastatin inhibits NF-kappaB signaling in intestinal epithelial cells and ameliorates acute murine colitis. Int Immunopharmacol. 2007;7(2):241-8.
355. Cho SJ, Kim JS, Kim JM, Lee JY, Jung HC, Song IS. Simvastatin induces apoptosis in human colon cancer cells and in tumor xenografts, and attenuates colitis-associated colon cancer in mice. Int J Cancer. 2008;123(4):951-7.
356. Suh N, Reddy BS, DeCastro A, Paul S, Lee HJ, Smolarek AK, et al. Combination of atorvastatin with sulindac or naproxen profoundly inhibits colonic adenocarcinomas by suppressing the p65/beta-catenin/cyclin D1 signaling pathway in rats. Cancer Prev Res (Phila). 2011;4(11):1895-902.
357. Banez LL, Klink JC, Jayachandran J, Lark AL, Gerber L, Hamilton RJ, et al. Association between statins and prostate tumor inflammatory infiltrate in men undergoing radical prostatectomy. Cancer Epidemiol Biomarkers Prev. 2010;19(3):722-8.
358. Malicki S, Winiarski M, Matlok M, Kostarczyk W, Guzdek A, Konturek PC. IL-6 and IL-8 responses of colorectal cancer in vivo and in vitro cancer cells subjected to simvastatin. J Physiol Pharmacol. 2009;60(4):141-6.
359. Wang XS, Williams LA, Krishnan S, Liao Z, Liu P, Mao L, et al. Serum sTNF-R1, IL-6, and the development of fatigue in patients with gastrointestinal cancer undergoing chemoradiation therapy. Brain Behav Immun. 2012;26(5):699-705.
360. Tønnesen H, Knigge U, Bülow S, Damm P, Fischerman K, Hesselfeldt P, et al. Effect of cimetidine on survival after gastric cancer. Lancet. 1988;2(8618):990-2.
361. Harvey VJ, Slevin ML, Dilloway MR, Clark PI, Johnston A, Lant AF. The influence of cimetidine on the pharmacokinetics of 5-fluorouracil. Br J Clin Pharmacol. 1984;18(3):421-30.
362. Adams WJ, Morris DL. Short-course cimetidine and survival with colorectal cancer. Lancet. 1994;344(8939-8940):1768-9.
363. Matsumoto S. Cimetidine and survival with colorectal cancer. Lancet. 1995;346(8967):115.
364. Matsumoto S, Imaeda Y, Umemoto S, Kobayashi K, Suzuki H, Okamoto T. Cimetidine increases survival of colorectal cancer patients with high levels of sialyl Lewis-X and sialyl Lewis-A epitope expression on tumour cells. Br J Cancer. 2002;86(2):161-7.

365. Svendsen LB, Ross C, Knigge U, Frederiksen HJ, Graversen P, Kjaergård J, et al. Cimetidine as an adjuvant treatment in colorectal cancer. A double-blind, randomized pilot study. Dis Colon Rectum. 1995;38(5):514-8.
366. Kelly MD, King J, Cherian M, Dwerryhouse SJ, Finlay IG, Adams WJ, et al. Randomized trial of preoperative cimetidine in patients with colorectal carcinoma with quantitative assessment of tumor-associated lymphocytes. Cancer. 1999;85(8):1658-63.
367. Nielsen HJ, Christensen IJ, Moesgaard F, Kehlet H, Group DRCCS. Ranitidine as adjuvant treatment in colorectal cancer. Br J Surg. 2002;89(11):1416-22.
368. Kapoor S, Pal S, Sahni P, Dattagupta S, Kanti Chattopadhyay T. Effect of pre-operative short course famotidine on tumor infiltrating lymphocytes in colorectal cancer: a double blind, placebo controlled, prospective randomized study. J Surg Res. 2005;129(2):172-5.
369. Deva S, Jameson M. Histamine type 2 receptor antagonists as adjuvant treatment for resected colorectal cancer. Cochrane Database Syst Rev. 2012(8):CD007814.
370. Adams WJ, Lawson JA, Morris DL. Cimetidine inhibits in vivo growth of human colon cancer and reverses histamine stimulated in vitro and in vivo growth. Gut. 1994;35(11):1632-6.
371. Cianchi F, Cortesini C, Schiavone N, Perna F, Magnelli L, Fanti E, et al. The role of cyclooxygenase-2 in mediating the effects of histamine on cell proliferation and vascular endothelial growth factor production in colorectal cancer. Clin Cancer Res. 2005;11(19 Pt 1):6807-15.
372. Masini E, Fabbroni V, Giannini L, Vannacci A, Messerini L, Perna F, et al. Histamine and histidine decarboxylase up-regulation in colorectal cancer: correlation with tumor stage. Inflamm Res. 2005;54 Suppl 1:S80-1.
373. Boer K, Helinger E, Helinger A, Pocza P, Pos Z, Demeter P, et al. Decreased expression of histamine H1 and H4 receptors suggests disturbance of local regulation in human colorectal tumours by histamine. Eur J Cell Biol. 2008;87(4):227-36.
374. Chen JS, Lin SY, Tso WL, Yeh GC, Lee WS, Tseng H, et al. Checkpoint kinase 1-mediated phosphorylation of Cdc25C and bad proteins are involved in antitumor effects of loratadine-induced G2/M phase cell-cycle arrest and apoptosis. Mol Carcinog. 2006;45(7):461-78.
375. Siegel JN, Schwartz A, Askenase PW, Gershon RK. T-cell suppression and contrasuppression induced by histamine H2 and H1 receptor agonists, respectively. Proc Natl Acad Sci U S A. 1982;79(16):5052-6.

376. Adams WJ, Morris DL. Pilot study--cimetidine enhances lymphocyte infiltration of human colorectal carcinoma: results of a small randomized control trial. Cancer. 1997;80(1):15-21.
377. Lin CY, Bai DJ, Yuan HY, Wang K, Yang GL, Hu MB, et al. Perioperative cimetidine administration promotes peripheral blood lymphocytes and tumor infiltrating lymphocytes in patients with gastrointestinal cancer: Results of a randomized controlled clinical trial. World J Gastroenterol. 2004;10(1):136-42.
378. Nielsen HJ, Moesgaard F, Hammer JH. Effect of ranitidine and low-dose interleukin-2 in vitro on NK-cell activity in peripheral blood from patients with liver metastases from colorectal cancer. Eur J Surg Oncol. 1995;21(5):526-30.
379. Adams WJ, Morris DL, Ross WB, Lubowski DZ, King DW, Peters L. Cimetidine preserves non-specific immune function after colonic resection for cancer. Aust N Z J Surg. 1994;64(12):847-52.
380. Rasmussen LA, Nielsen HJ, Sorensen S, Sorensen C, Rasmussen R, Moesgaard F, et al. Ranitidine reduces postoperative interleukin-6 induced C-reactive protein synthesis. J Am Coll Surg. 1995;181(2):138-44.
381. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-917.
382. Fleming ID, American Joint Committee on Cancer., American Cancer Society., American College of Surgeons. AJCC cancer staging manual. 5th ed / ed. Philadelphia: Lippincott-Raven; 1997. xv, 294 p. p.
383. Horgan PG, McMillan DC. Surgeons and selection of adjuvant therapy for node-negative colonic cancer. Br J Surg. 2010;97(10):1459-60.
384. McMillan DC. The systemic inflammation-based Glasgow Prognostic Score: a decade of experience in patients with cancer. Cancer Treat Rev. 2013;39(5):534-40.
385. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. Crit Rev Oncol Hematol. 2013;88(1):218-30.
386. Proctor MJ, Morrison DS, Talwar D, Balmer SM, Fletcher CD, O'Reilly DS, et al. A comparison of inflammation-based prognostic scores in patients with cancer. A Glasgow Inflammation Outcome Study. Eur J Cancer. 2011;47(17):2633-41.
387. Leitch EF, Chakrabarti M, Crozier JE, McKee RF, Anderson JH, Horgan PG, et al. Comparison of the prognostic value of selected markers of the systemic inflammatory response in patients with colorectal cancer. Br J Cancer. 2007;97(9):1266-70.

388. Guthrie GJ, Roxburgh CS, Farhan-Alanie OM, Horgan PG, McMillan DC. Comparison of the prognostic value of longitudinal measurements of systemic inflammation in patients undergoing curative resection of colorectal cancer. Br J Cancer. 2013;109(1):24-8.
389. Choi KW, Hong SW, Chang YG, Lee WY, Lee B, Paik IW, et al. Inflammation-based score (Glasgow prognostic score) as an independent prognostic factor in colorectal cancer patients. Ann Surg Treat Res. 2014;86(6):309-13.
390. Powell AG, Wallace R, McKee RF, Anderson JH, Going JJ, Edwards J, et al. The relationship between tumour site, clinicopathological characteristics and cancer-specific survival in patients undergoing surgery for colorectal cancer. Colorectal Dis. 2012;14(12):1493-9.
391. Richards CH, Leitch EF, Horgan PG, Anderson JH, McKee RF, McMillan DC. The relationship between patient physiology, the systemic inflammatory response and survival in patients undergoing curative resection of colorectal cancer. Br J Cancer. 2010;103(9):1356-61.
392. Roxburgh CS, Platt JJ, Leitch EF, Kinsella J, Horgan PG, McMillan DC. Relationship between preoperative comorbidity, systemic inflammatory response, and survival in patients undergoing curative resection for colorectal cancer. Ann Surg Oncol. 2011;18(4):997-1005.
393. Mohri Y, Miki C, Kobayashi M, Okita Y, Inoue M, Uchida K, et al. Correlation between preoperative systemic inflammation and postoperative infection in patients with gastrointestinal cancer: a multicenter study. Surg Today. 2014;44(5):859-67.
394. Roxburgh C, McDonald A, Salmond J, Oien K, Anderson J, McKee R, et al. Adjuvant chemotherapy for resected colon cancer: comparison of the prognostic value of tumour and patient related factors. Int J Colorectal Dis. 2011;26(4):483-92.
395. Michigan A, Johnson TV, Master VA. Preoperative C-reactive protein level adjusted for comorbidities and lifestyle factors predicts overall mortality in localized renal cell carcinoma. Mol Diagn Ther. 2011;15(4):229-34.
396. Slaviero KA, Clarke SJ, Rivory LP. Inflammatory response: an unrecognised source of variability in the pharmacokinetics and pharmacodynamics of cancer chemotherapy. Lancet Oncol. 2003;4(4):224-32.
397. Sharma R, Zucknick M, London R, Kacevska M, Liddle C, Clarke SJ. Systemic inflammatory response predicts prognosis in patients with advanced-stage colorectal cancer. Clin Colorectal Cancer. 2008;7(5):331-7.
398. McAllister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. Nat Cell Biol. 2014;16(8):717-27.

399. Douglas E, McMillan DC. Towards a simple objective framework for the investigation and treatment of cancer cachexia: the Glasgow Prognostic Score. Cancer Treat Rev. 2014;40(6):685-91.
400. Roxburgh CS, McMillan DC. Cancer and systemic inflammation: treat the tumour and treat the host. Br J Cancer. 2014;110(6):1409-12.
401. Khera A, McGuire DK, Murphy SA, Stanek HG, Das SR, Vongpatanasin W, et al. Race and gender differences in C-reactive protein levels. J Am Coll Cardiol. 2005;46(3):464-9.
402. Woloshin S, Schwartz LM. Distribution of C-reactive protein values in the United States. N Engl J Med. 2005;352(15):1611-3.
403. Nazmi A, Victora CG. Socioeconomic and racial/ethnic differentials of C-reactive protein levels: a systematic review of population-based studies. BMC Public Health. 2007;7:212.
404. Yamada S, Gotoh T, Nakashima Y, Kayaba K, Ishikawa S, Nago N, et al. Distribution of serum C-reactive protein and its association with atherosclerotic risk factors in a Japanese population : Jichi Medical School Cohort Study. Am J Epidemiol. 2001;153(12):1183-90.
405. Lakoski SG, Cushman M, Palmas W, Blumenthal R, D'Agostino RB, Jr., Herrington DM. The relationship between blood pressure and C-reactive protein in the Multi-Ethnic Study of Atherosclerosis (MESA). J Am Coll Cardiol. 2005;46(10):1869-74.
406. McDade TW, Rutherford JN, Adair L, Kuzawa C. Population differences in associations between C-reactive protein concentration and adiposity: comparison of young adults in the Philippines and the United States. Am J Clin Nutr. 2009;89(4):1237-45.
407. Watanabe T, Itabashi M, Shimada Y, Tanaka S, Ito Y, Ajioka Y, et al. Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2014 for treatment of colorectal cancer. International journal of clinical oncology. 2015;20(2):207-39.
408. Nagtegaal ID, Tot T, Jayne DG, McShane P, Nihlberg A, Marshall HC, et al. Lymph nodes, tumor deposits, and TNM: are we getting better? J Clin Oncol. 2011;29(18):2487-92.
409. Ueno H, Mochizuki H, Akagi Y, Kusumi T, Yamada K, Ikegami M, et al. Optimal colorectal cancer staging criteria in TNM classification. J Clin Oncol. 2012;30(13):1519-26.
410. Doyle VJ, Bateman AC. Colorectal cancer staging using TNM 7: is it time to use this new staging system? J Clin Pathol. 2012;65(4):372-4.

411. Kelley-Hedgepeth A, Lloyd-Jones DM, Colvin A, Matthews KA, Johnston J, Sowers MR, et al. Ethnic differences in C-reactive protein concentrations. Clin Chem. 2008;54(6):1027-37.
412. Hage FG, Szalai AJ. C-reactive protein gene polymorphisms, C-reactive protein blood levels, and cardiovascular disease risk. J Am Coll Cardiol. 2007;50(12):1115-22.
413. Carlson CS, Aldred SF, Lee PK, Tracy RP, Schwartz SM, Rieder M, et al. Polymorphisms within the C-reactive protein (CRP) promoter region are associated with plasma CRP levels. Am J Hum Genet. 2005;77(1):64-77.
414. Dorajoo R, Li R, Ikram MK, Liu J, Froguel P, Lee J, et al. Are C-reactive protein associated genetic variants associated with serum levels and retinal markers of microvascular pathology in Asian populations from Singapore? PloS one. 2013;8(7):e67650.
415. Kocarnik JM, Pendergrass SA, Carty CL, Pankow JS, Schumacher FR, Cheng I, et al. Multiancestral analysis of inflammation-related genetic variants and C-reactive protein in the population architecture using genomics and epidemiology study. Circ Cardiovasc Genet. 2014;7(2):178-88.
416. Solheim TS, Fayers PM, Fladvad T, Tan B, Skorpen F, Fearon K, et al. Is there a genetic cause for cancer cachexia? - a clinical validation study in 1797 patients. Br J Cancer. 2011;105(8):1244-51.
417. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. JAMA. 1999;282(22):2131-5.
418. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2014;384(9945):766-81.
419. Penston J. Should we use total mortality rather than cancer specific mortality to judge cancer screening programmes? Yes. BMJ. 2011;343:d6395.
420. Saridaki Z, Souglakos J, Georgoulias V. Prognostic and predictive significance of MSI in stages II/III colon cancer. World J Gastroenterol. 2014;20(22):6809-14.
421. Summary of the Results of Population Census of Japan 2010: Statitistics Bureau, Ministry of Intenral Affairs and Communications; 2010 [Available from: http://www.stat.go.jp/english/data/kokusei/2010/final_en/pdf/summary.pdf.
422. Freeman MR, Li Q, Chung LW. Can stroma reaction predict cancer lethality? Clin Cancer Res. 2013;19(18):4905-7.

423. Liu R, Li J, Xie K, Zhang T, Lei Y, Chen Y, et al. FGFR4 Promotes Stroma-Induced Epithelial-to-Mesenchymal Transition in Colorectal Cancer. Cancer Res. 2013;73(19):5926-35.
424. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. J Pathol. 2003;200(4):429-47.
425. Petty RD, Samuel LM, Murray GI, MacDonald G, O'Kelly T, Loudon M, et al. APRIL is a novel clinical chemo-resistance biomarker in colorectal adenocarcinoma identified by gene expression profiling. BMC cancer. 2009;9:434.
426. Verset L, Tommelein J, Moles Lopez X, Decaestecker C, Boterberg T, De Vlieghere E, et al. Impact of neoadjuvant therapy on cancer-associated fibroblasts in rectal cancer. Radiother Oncol. 2015.
427. Dekker TJ, van de Velde CJ, van Pelt GW, Kroep JR, Julien JP, Smit VT, et al. Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). Breast Cancer Res Treat. 2013;139(2):371-9.
428. Courrech Staal EF, Wouters MW, van Sandick JW, Takkenberg MM, Smit VT, Junggeburt JM, et al. The stromal part of adenocarcinomas of the oesophagus: does it conceal targets for therapy? Eur J Cancer. 2010;46(4):720-8.
429. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol. 2007;29(5):387-98.
430. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24(1):179-85.
431. Mesker WE, Liefers GJ, Junggeburt JM, van Pelt GW, Alberici P, Kuppen PJ, et al. Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cell Oncol. 2009;31(3):169-78.
432. Roxburgh CS, McMillan DC, Richards CH, Atwan M, Anderson JH, Harvey T, et al. The clinical utility of the combination of T stage and venous invasion to predict survival in patients undergoing surgery for colorectal cancer. Ann Surg. 2014;259(6):1156-65.
433. Richards CH, Roxburgh CS, Powell AG, Foulis AK, Horgan PG, McMillan DC. The clinical utility of the local inflammatory response in colorectal cancer. Eur J Cancer. 2014;50(2):309-19.
434. Pollheimer MJ, Kornprat P, Lindtner RA, Harbaum L, Schlemmer A, Rehak P, et al. Tumor necrosis is a new promising prognostic factor in colorectal cancer. Hum Pathol. 2010;41(12):1749-57.

435. Koshida Y, Kuranami M, Watanabe M. Interaction between stromal fibroblasts and colorectal cancer cells in the expression of vascular endothelial growth factor. J Surg Res. 2006;134(2):270-7.
436. Moserle L, Casanovas O. Anti-angiogenesis and metastasis: a tumour and stromal cell alliance. J Intern Med. 2013;273(2):128-37.
437. Koukourakis MI, Giatromanolaki A, Harris AL, Sivridis E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. Cancer Res. 2006;66(2):632-7.
438. Guthrie GJ, Roxburgh CS, Richards CH, Horgan PG, McMillan DC. Circulating IL-6 concentrations link tumour necrosis and systemic and local inflammatory responses in patients undergoing resection for colorectal cancer. Br J Cancer. 2013;109(1):131-7.
439. Zlobec I, Lugli A. Invasive front of colorectal cancer: dynamic interface of pro-/anti-tumor factors. World J Gastroenterol. 2009;15(47):5898-906.
440. Hemmings C. Is carcinoma a mesenchymal disease? The role of the stromal microenvironment in carcinogenesis. Pathology. 2013;45(4):371-81.
441. Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, et al. Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res. 2007;13(7):2082-90.
442. Engels B, Rowley DA, Schreiber H. Targeting stroma to treat cancers. Semin Cancer Biol. 2012;22(1):41-9.
443. Hartmann N, Giese NA, Giese T, Poschke I, Offringa R, Werner J, et al. Prevailing role of contact guidance in intrastromal T-cell trapping in human pancreatic cancer. Clin Cancer Res. 2014;20(13):3422-33.
444. Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G. Prognostic and predictive impact of intra- and peritumoral immune infiltrates. Cancer Res. 2011;71(17):5601-5.
445. De Smedt L, Lemahieu J, Palmans S, Govaere O, Tousseyn T, Van Cutsem E, et al. Microsatellite instable vs stable colon carcinomas: analysis of tumour heterogeneity, inflammation and angiogenesis. Br J Cancer. 2015;113(3):500-9.
446. Roxburgh CS, Richards CH, Macdonald AI, Powell AG, McGlynn LM, McMillan DC, et al. The in situ local immune response, tumour senescence and proliferation in colorectal cancer. Br J Cancer. 2013;109(8):2207-16.
447. Watt DG, Martin JC, Park JH, Horgan PG, McMillan DC. Neutrophil count is the most important prognostic component of the differential white cell count in patients undergoing elective surgery for colorectal cancer. Am J Surg. 2015;210(1):24-30.

448. Watt DG, Proctor MJ, Park JH, Horgan PG, McMillan DC. The Neutrophil-Platelet Score (NPS) Predicts Survival in Primary Operable Colorectal Cancer and a Variety of Common Cancers. PloS one. 2015;10(11):e0142159.
449. Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ES. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol. 2003;16(1):79-84.
450. Jourdan F, Sebbagh N, Comperat E, Mourra N, Flahault A, Olschwang S, et al. Tissue microarray technology: validation in colorectal carcinoma and analysis of p53, hMLH1, and hMSH2 immunohistochemical expression. Virchows Arch. 2003;443(2):115-21.
451. Vayrynen JP, Sajanti SA, Klintrup K, Makela J, Herzig KH, Karttunen TJ, et al. Characteristics and significance of colorectal cancer associated lymphoid reaction. Int J Cancer. 2014;134(9):2126-35.
452. Vayrynen JP, Tuomisto A, Klintrup K, Makela J, Karttunen TJ, Makinen MJ. Detailed analysis of inflammatory cell infiltration in colorectal cancer. Br J Cancer. 2013;109(7):1839-47.
453. Maby P, Tougeron D, Hamieh M, Mlecnik B, Kora H, Bindea G, et al. Correlation between Density of CD8+ T-cell Infiltrate in Microsatellite Unstable Colorectal Cancers and Frameshift Mutations: A Rationale for Personalized Immunotherapy. Cancer Res. 2015;75(17):3446-55.
454. Pine JK, Morris E, Hutchins GG, West NP, Jayne DG, Quirke P, et al. Systemic neutrophil-to-lymphocyte ratio in colorectal cancer: the relationship to patient survival, tumour biology and local lymphocytic response to tumour. Br J Cancer. 2015;113(2):204-11.
455. Missiaglia E, Jacobs B, D'Ario G, Di Narzo AF, Soneson C, Budinska E, et al. Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. Ann Oncol. 2014;25(10):1995-2001.
456. Fuseya C, Horiuchi A, Hayashi A, Suzuki A, Miyamoto T, Hayashi T, et al. Involvement of pelvic inflammation-related mismatch repair abnormalities and microsatellite instability in the malignant transformation of ovarian endometriosis. Hum Pathol. 2012;43(11):1964-72.
457. Tseng-Rogenski SS, Hamaya Y, Choi DY, Carethers JM. Interleukin 6 alters localization of hMSH3, leading to DNA mismatch repair defects in colorectal cancer cells. Gastroenterology. 2015;148(3):579-89.
458. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-64.

459. Sinicrope FA, Rego RL, Ansell SM, Knutson KL, Foster NR, Sargent DJ. Intraepithelial effector (CD3+)/regulatory (FoxP3+) T-cell ratio predicts a clinical outcome of human colon carcinoma. Gastroenterology. 2009;137(4):1270-9.
460. Dahlin AM, Henriksson ML, Van Guelpen B, Stenling R, Oberg A, Rutegard J, et al. Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. Mod Pathol. 2011;24(5):671-82.
461. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. J Mol Diagn. 2008;10(4):293-300.
462. Lindor NM, Burgart LJ, Leontovich O, Goldberg RM, Cunningham JM, Sargent DJ, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol. 2002;20(4):1043-8.
463. Hendriks Y, Franken P, Dierssen JW, De Leeuw W, Wijnen J, Dreef E, et al. Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors. Am J Pathol. 2003;162(2):469-77.
464. Forrest R, Guthrie GJ, Orange C, Horgan PG, McMillan DC, Roxburgh CS. Comparison of visual and automated assessment of tumour inflammatory infiltrates in patients with colorectal cancer. Eur J Cancer. 2014;50(3):544-52.
465. Kantola T, Klintrup K, Vayrynen JP, Vornanen J, Bloigu R, Karhu T, et al. Stage-dependent alterations of the serum cytokine pattern in colorectal carcinoma. Br J Cancer. 2012;107(10):1729-36.
466. Guo Y, Xu F, Lu T, Duan Z, Zhang Z. Interleukin-6 signaling pathway in targeted therapy for cancer. Cancer Treat Rev. 2012;38(7):904-10.
467. Nagasaki T, Hara M, Nakanishi H, Takahashi H, Sato M, Takeyama H. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. Br J Cancer. 2014;110(2):469-78.
468. Huynh PT, Beswick EJ, Coronado YA, Johnson P, O'Connell MR, Watts T, et al. CD90(+) stromal cells are the major source of IL-6, which supports cancer stem-like cells and inflammation in colorectal cancer. Int J Cancer. 2016;138(8):1971-81.
469. Wang SW, Sun YM. The IL-6/JAK/STAT3 pathway: potential therapeutic strategies in treating colorectal cancer (Review). Int J Oncol. 2014;44(4):1032-40.
470. Taniguchi K, Karin M. IL-6 and related cytokines as the critical lynchpins between inflammation and cancer. Semin Immunol. 2014;26(1):54-74.

471. Li MX, Bi XY, Huang Z, Zhao JJ, Han Y, Li ZY, et al. Prognostic Role of Phospho-STAT3 in Patients with Cancers of the Digestive System: A Systematic Review and Meta-Analysis. PloS one. 2015;10(5):e0127356.
472. Kirkegaard T, Edwards J, Tovey S, McGlynn LM, Krishna SN, Mukherjee R, et al. Observer variation in immunohistochemical analysis of protein expression, time for a change? Histopathology. 2006;48(7):787-94.
473. Morikawa T, Baba Y, Yamauchi M, Kuchiba A, Nosho K, Shima K, et al. STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. Clin Cancer Res. 2011;17(6):1452-62.
474. Denley SM, Jamieson NB, McCall P, Oien KA, Morton JP, Carter CR, et al. Activation of the IL-6R/Jak/stat pathway is associated with a poor outcome in resected pancreatic ductal adenocarcinoma. J Gastrointest Surg. 2013;17(5):887-98.
475. Nguyen AV, Wu YY, Liu Q, Wang D, Nguyen S, Loh R, et al. STAT3 in epithelial cells regulates inflammation and tumor progression to malignant state in colon. Neoplasia. 2013;15(9):998-1008.
476. O'Toole A, Michielsen AJ, Nolan B, Tosetto M, Sheahan K, Mulcahy HE, et al. Tumour microenvironment of both early- and late-stage colorectal cancer is equally immunosuppressive. Br J Cancer. 2014;111(5):927-32.
477. Yu H, Liu Y, McFarland BC, Deshane JS, Hurst DR, Ponnazhagan S, et al. SOCS3 Deficiency in Myeloid Cells Promotes Tumor Development: Involvement of STAT3 Activation and Myeloid-Derived Suppressor Cells. Cancer Immunol Res. 2015.
478. Griesinger AM, Josephson RJ, Donson AM, Mulcahy Levy JM, Amani V, Birks DK, et al. Interleukin-6/STAT3 pathway signaling drives an inflammatory phenotype in Group A ependymoma. Cancer Immunol Res. 2015;3(10):1165-74.
479. Chen ZY, Raghav K, Lieu CH, Jiang ZQ, Eng C, Vauthey JN, et al. Cytokine profile and prognostic significance of high neutrophil-lymphocyte ratio in colorectal cancer. Br J Cancer. 2015;112(6):1088-97.
480. Sehgal PB. STAT-signalling through the cytoplasmic compartment: consideration of a new paradigm. Cell Signal. 2000;12(8):525-35.
481. Germain D, Frank DA. Targeting the cytoplasmic and nuclear functions of signal transducers and activators of transcription 3 for cancer therapy. Clin Cancer Res. 2007;13(19):5665-9.
482. Bonetto A, Aydogdu T, Kunzevitzky N, Guttridge DC, Khuri S, Koniaris LG, et al. STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. PloS one. 2011;6(7):e22538.

483. Lim CP, Cao X. Serine phosphorylation and negative regulation of Stat3 by JNK. J Biol Chem. 1999;274(43):31055-61.
484. Liu L, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. Proc Natl Acad Sci U S A. 2005;102(23):8150-5.
485. Bak Y, Kwon T, Bak IS, Hong J, Yu DY, Yoon DY. IL-32theta inhibits stemness and epithelial-mesenchymal transition of cancer stem cells via the STAT3 pathway in colon cancer. Oncotarget. 2016;7(6):7307-17.
486. Wang S, Liu Z, Wang L, Zhang X. NF-kappaB signaling pathway, inflammation and colorectal cancer. Cell Mol Immunol. 2009;6(5):327-34.
487. De Simone V, Franze E, Ronchetti G, Colantoni A, Fantini MC, Di Fusco D, et al. Th17-type cytokines, IL-6 and TNF-alpha synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. Oncogene. 2015;34(27):3493-503.
488. Sansone P, Bromberg J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. J Clin Oncol. 2012;30(9):1005-14.
489. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annu Rev Med. 2015;66:311-28.
490. Diakos CI, Charles KA, McMillan DC, Clarke SJ. Cancer-related inflammation and treatment effectiveness. Lancet Oncol. 2014;15(11):e493-503.
491. Hurwitz HI, Uppal N, Wagner SA, Bendell JC, Beck JT, Wade SM, 3rd, et al. Randomized, Double-Blind, Phase II Study of Ruxolitinib or Placebo in Combination With Capecitabine in Patients With Metastatic Pancreatic Cancer for Whom Therapy With Gemcitabine Has Failed. J Clin Oncol. 2015;33(34):4039-47.
492. Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015;21(11):1350-6.
493. Corcoran RB, Contino G, Deshpande V, Tzatsos A, Conrad C, Benes CH, et al. STAT3 plays a critical role in KRAS-induced pancreatic tumorigenesis. Cancer Res. 2011;71(14):5020-9.
494. Dobi E, Monnien F, Kim S, Ivanaj A, N'Guyen T, Demarchi M, et al. Impact of STAT3 phosphorylation on the clinical effectiveness of anti-EGFR-based therapy in patients with metastatic colorectal cancer. Clin Colorectal Cancer. 2013;12(1):28-36.
495. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. Nat Protoc. 2007;2(8):1849-61.

496. Mansouri D, McMillan DC, Crighton EM, Horgan PG. Screening for colorectal cancer: what is the impact on the determinants of outcome? Crit Rev Oncol Hematol. 2013;85(3):342-9.
497. Church D, Midgley R, Kerr D. Biomarkers in early-stage colorectal cancer: ready for prime time? Digestive diseases. 2012;30 Suppl 2:27-33.
498. Angell HK, Gray N, Womack C, Pritchard DI, Wilkinson RW, Cumberbatch M. Digital pattern recognition-based image analysis quantifies immune infiltrates in distinct tissue regions of colorectal cancer and identifies a metastatic phenotype. Br J Cancer. 2013;109(6):1618-24.
499. Sinicrope FA, Shi Q, Smyrk TC, Thibodeau SN, Dienstmann R, Guinney J, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. Gastroenterology. 2015;148(1):88-99.
500. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39(4):782-95.
501. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J Pathol. 2014;232(2):199-209.
502. Chrysanthopoulou A, Mitroulis I, Apostolidou E, Arelaki S, Mikroulis D, Konstantinidis T, et al. Neutrophil extracellular traps promote differentiation and function of fibroblasts. J Pathol. 2014;233(3):294-307.
503. Koelzer VH, Dawson H, Andersson E, Karamitopoulou E, Masucci GV, Lugli A, et al. Active immunosurveillance in the tumor microenvironment of colorectal cancer is associated with low frequency tumor budding and improved outcome. Transl Res. 2015;166(2):207-17.
504. Mitrovic B, Schaeffer DF, Riddell RH, Kirsch R. Tumor budding in colorectal carcinoma: time to take notice. Mod Pathol. 2012;25(10):1315-25.
505. Max N, Harbaum L, Pollheimer MJ, Lindtner RA, Kornprat P, Langner C. Tumour budding with and without admixed inflammation: two different sides of the same coin? Br J Cancer. 2016;114(4):368-71.
506. Vayrynen SA, Vayrynen JP, Klintrup K, Makela J, Karttunen TJ, Tuomisto A, et al. Clinical impact and network of determinants of tumour necrosis in colorectal cancer. Br J Cancer. 2016;114(12):1334-42.
507. Swinson DE, Jones JL, Richardson D, Cox G, Edwards JG, O'Byrne KJ. Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. Lung Cancer. 2002;37(3):235-40.

508. Gunderson LL, Jessup JM, Sargent DJ, Greene FL, Stewart AK. Revised TN categorization for colon cancer based on national survival outcomes data. J Clin Oncol. 2010;28(2):264-71.
509. Li J, Yi CH, Hu YT, Li JS, Yuan Y, Zhang SZ, et al. TNM Staging of Colorectal Cancer Should be Reconsidered According to Weighting of the T Stage: Verification Based on a 25-Year Follow-Up. Medicine (Baltimore). 2016;95(6):e2711.
510. Koelzer VH, Lugli A. The tumor border configuration of colorectal cancer as a histomorphological prognostic indicator. Front Oncol. 2014;4:29.
511. van Wyk HC, Park J, Roxburgh C, Horgan P, Foulis A, McMillan DC. The role of tumour budding in predicting survival in patients with primary operable colorectal cancer: a systematic review. Cancer Treat Rev. 2015;41(2):151-9.
512. Anitei MG, Zeitoun G, Mlecnik B, Marliot F, Haicheur N, Todosi AM, et al. Prognostic and predictive values of the immunoscore in patients with rectal cancer. Clin Cancer Res. 2014;20(7):1891-9.
513. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A. Tumor associated macrophages and neutrophils in tumor progression. J Cell Physiol. 2013;228(7):1404-12.
514. Turner N, Wong HL, Templeton A, Tripathy S, Rogers TW, Croxford M, et al. Analysis of local chronic inflammatory cell infiltrate combined with systemic inflammation improves prognostication in stage II colon cancer independent of standard clinicopathologic criteria. Int J Cancer. 2015.
515. Cole BF, Logan RF, Halabi S, Benamouzig R, Sandler RS, Grainge MJ, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. J Natl Cancer Inst. 2009;101(4):256-66.
516. Ait Ouakrim D, Dashti SG, Chau R, Buchanan DD, Clendenning M, Rosty C, et al. Aspirin, Ibuprofen, and the Risk of Colorectal Cancer in Lynch Syndrome. J Natl Cancer Inst. 2015;107(9).
517. Wang Y, Zhang FC, Wang YJ. The efficacy and safety of non-steroidal anti-inflammatory drugs in preventing the recurrence of colorectal adenoma: a meta-analysis and systematic review of randomized trials. Colorectal Dis. 2015;17(3):188-96.
518. Mansouri D, McMillan DC, Roxburgh CS, Crighton EM, Horgan PG. The impact of aspirin, statins and ACE-inhibitors on the presentation of colorectal neoplasia in a colorectal cancer screening programme. Br J Cancer. 2013;109(1):249-56.
519. Ye X, Fu J, Yang Y, Chen S. Dose-risk and duration-risk relationships between aspirin and colorectal cancer: a meta-analysis of published cohort studies. PloS one. 2013;8(2):e57578.

520. McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. Eur J Cancer. 2013;49(5):1049-57.
521. Li P, Wu H, Zhang H, Shi Y, Xu J, Ye Y, et al. Aspirin use after diagnosis but not prediagnosis improves established colorectal cancer survival: a meta-analysis. Gut. 2015;64(9):1419-25.
522. Cai H, Zhang G, Wang Z, Luo Z, Zhou X. Relationship between the use of statins and patient survival in colorectal cancer: a systematic review and meta-analysis. PloS one. 2015;10(6):e0126944.
523. Cardwell CR, Hicks BM, Hughes C, Murray LJ. Statin use after colorectal cancer diagnosis and survival: a population-based cohort study. J Clin Oncol. 2014;32(28):3177-83.
524. Liu Y, Tang W, Wang J, Xie L, Li T, He Y, et al. Association between statin use and colorectal cancer risk: a meta-analysis of 42 studies. Cancer Causes Control. 2014;25(2):237-49.
525. Reimers MS, Bastiaannet E, Langley RE, van Eijk R, van Vlierberghe RL, Lemmens VE, et al. Expression of HLA class I antigen, aspirin use, and survival after a diagnosis of colon cancer. JAMA Intern Med. 2014;174(5):732-9.
526. Domingo E, Church DN, Sieber O, Ramamoorthy R, Yanagisawa Y, Johnstone E, et al. Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. J Clin Oncol. 2013;31(34):4297-305.
527. Haynes SR, Lawler PG. An assessment of the consistency of ASA physical status classification allocation. Anaesthesia. 1995;50(3):195-9.
528. Richards CH, Platt JJ, Anderson JH, McKee RF, Horgan PG, McMillan DC. The impact of perioperative risk, tumor pathology and surgical complications on disease recurrence following potentially curative resection of colorectal cancer. Ann Surg. 2011;254(1):83-9.
529. Sankar A, Johnson SR, Beattie WS, Tait G, Wijeysundera DN. Reliability of the American Society of Anesthesiologists physical status scale in clinical practice. Br J Anaesth. 2014;113(3):424-32.
530. Cuvillon P, Nouvellon E, Marret E, Albaladejo P, Fortier LP, Fabbro-Perray P, et al. American Society of Anesthesiologists' physical status system: a multicentre Francophone study to analyse reasons for classification disagreement. Eur J Anaesthesiol. 2011;28(10):742-7.

531. Aronson D, Bartha P, Zinder O, Kerner A, Markiewicz W, Avizohar O, et al. Obesity is the major determinant of elevated C-reactive protein in subjects with the metabolic syndrome. Int J Obes Relat Metab Disord. 2004;28(5):674-9.
532. Jonsson F, Yin L, Lundholm C, Smedby KE, Czene K, Pawitan Y. Low-dose aspirin use and cancer characteristics: a population-based cohort study. Br J Cancer. 2013;109(7):1921-5.
533. Pawitan Y, Yin L, Setiawan A, Auer G, Smedby KE, Czene K. Distinct effects of anti-inflammatory and anti-thrombotic drugs on cancer characteristics at diagnosis. Eur J Cancer. 2015;51(6):751-7.
534. Herbert K, Kerr R, Kerr DJ, Church DN. Are NSAIDs Coming Back to Colorectal Cancer Therapy or Not? Curr Colorectal Cancer Rep. 2014;10(4):363-71.
535. Tian Y, Ye Y, Gao W, Chen H, Song T, Wang D, et al. Aspirin promotes apoptosis in a murine model of colorectal cancer by mechanisms involving downregulation of IL-6-STAT3 signaling pathway. Int J Colorectal Dis. 2011;26(1):13-22.
536. Guo H, Liu J, Ben Q, Qu Y, Li M, Wang Y, et al. The aspirin-induced long non-coding RNA OLA1P2 blocks phosphorylated STAT3 homodimer formation. Genome Biol. 2016;17(1):24.
537. Slattery ML, Lundgreen A, Hines LM, Torres-Mejia G, Wolff RK, Stern MC, et al. Genetic variation in the JAK/STAT/SOCS signaling pathway influences breast cancer-specific mortality through interaction with cigarette smoking and use of aspirin/NSAIDs: the Breast Cancer Health Disparities Study. Breast Cancer Res Treat. 2014;147(1):145-58.
538. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;20(5):576-90.
539. Marino M, Scuderi F, Ponte E, Maiuri MT, De Cristofaro R, Provenzano C, et al. Novel path to IL-6 trans-signaling through thrombin-induced soluble IL-6 receptor release by platelets. J Biol Regul Homeost Agents. 2013;27(3):841-52.
540. Gremmel T, Perkmann T, Kopp CW, Seidinger D, Eichelberger B, Koppensteiner R, et al. Interleukin-6 and asymmetric dimethylarginine are associated with platelet activation after percutaneous angioplasty with stent implantation. PloS one. 2015;10(3):e0122586.
541. Zanders MM, van Herk-Sukel MP, Vissers PA, Herings RM, Haak HR, van de Poll-Franse LV. Are metformin, statin and aspirin use still associated with overall mortality among colorectal cancer patients with diabetes if adjusted for one another? Br J Cancer. 2015;113(3):403-10.

542. Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer. 2005;5(12):930-42.
543. A phase III, double-blind, placebo-controlled, randomised trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common non-metastatic solid tumours. 2014 [13th November 2014:[Available from: http://www.ctu.mrc.ac.uk/13391/13399/18269/addaspirin_protocol.
544. Morant SV, McMahon AD, Cleland JG, Davey PG, MacDonald TM. Cardiovascular prophylaxis with aspirin: costs of supply and management of upper gastrointestinal and renal toxicity. Br J Clin Pharmacol. 2004;57(2):188-98.
545. Yasuda K, Nirei T, Sunami E, Nagawa H, Kitayama J. Density of CD4(+) and CD8(+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. Radiat Oncol. 2011;6:49.
546. Teng F, Meng X, Kong L, Mu D, Zhu H, Liu S, et al. Tumor-infiltrating lymphocytes, forkhead box P3, programmed death ligand-1, and cytotoxic T lymphocyte-associated antigen-4 expressions before and after neoadjuvant chemoradiation in rectal cancer. Transl Res. 2015;166(6):721-32 e1.
547. Shinto E, Hase K, Hashiguchi Y, Sekizawa A, Ueno H, Shikina A, et al. CD8+ and FOXP3+ tumor-infiltrating T cells before and after chemoradiotherapy for rectal cancer. Ann Surg Oncol. 2014;21 Suppl 3:S414-21.
548. Klemm F, Joyce JA. Microenvironmental regulation of therapeutic response in cancer. Trends Cell Biol. 2015;25(4):198-213.
549. Frey B, Rubner Y, Kulzer L, Werthmoller N, Weiss EM, Fietkau R, et al. Antitumor immune responses induced by ionizing irradiation and further immune stimulation. Cancer Immunol Immunother. 2014;63(1):29-36.
550. Roxburgh CS, McMillan DC. Therapeutics targeting innate immune/inflammatory responses through the interleukin-6/JAK/STAT signal transduction pathway in patients with cancer. Transl Res. 2016;167(1):61-6.
551. van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, et al. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch. 2008;453(5):417-31.
552. Saridaki Z, Saegart X, De Vriendt V, Hatzidaki D, Palmans S, De Smedt L, et al. KRAS, NRAS, BRAF mutation comparison of endoscopic and surgically removed primary CRC paired samples: is endoscopy biopsy material adequate for molecular evaluation? Br J Cancer. 2015;113(6):914-20.

553. Courrech Staal EF, Smit VT, van Velthuysen ML, Spitzer-Naaykens JM, Wouters MW, Mesker WE, et al. Reproducibility and validation of tumour stroma ratio scoring on oesophageal adenocarcinoma biopsies. Eur J Cancer. 2011;47(3):375-82.
554. Van Ness PH, Towle VR, Juthani-Mehta M. Testing measurement reliability in older populations: methods for informed discrimination in instrument selection and application. J Aging Health. 2008;20(2):183-97.
555. van der Post RS, van der Laak JA, Sturm B, Clarijs R, Schaafsma HE, van Krieken JH, et al. The evaluation of colon biopsies using virtual microscopy is reliable. Histopathology. 2013;63(1):114-21.
556. Loughrey MB, Kelly PJ, Houghton OP, Coleman HG, Houghton JP, Carson A, et al. Digital slide viewing for primary reporting in gastrointestinal pathology: a validation study. Virchows Arch. 2015;467(2):137-44.
557. Mohammed ZM, Going JJ, McMillan DC, Orange C, Mallon E, Doughty JC, et al. Comparison of visual and automated assessment of HER2 status and their impact on outcome in primary operable invasive ductal breast cancer. Histopathology. 2012;61(4):675-84.
558. Polanska UM, Orimo A. Carcinoma-associated fibroblasts: non-neoplastic tumour-promoting mesenchymal cells. J Cell Physiol. 2013;228(8):1651-7.
559. Heinemann V, Reni M, Ychou M, Richel DJ, Macarulla T, Ducreux M. Tumour-stroma interactions in pancreatic ductal adenocarcinoma: rationale and current evidence for new therapeutic strategies. Cancer Treat Rev. 2014;40(1):118-28.
560. Rozek LS, Schmit SL, Greenson JK, Tomsho LP, Rennert HS, Rennert G, et al. Tumor-Infiltrating Lymphocytes, Crohn's-Like Lymphoid Reaction, and Survival From Colorectal Cancer. J Natl Cancer Inst. 2016;108(8).
561. Galon J, Mlecnik B, Marliot F, Ou F, Bifulco C, Lugli A, et al. Validation of the Immunoscore (IM) as a prognostic marker in stage I/II/III colon cancer: Results of a worldwide consortium-based analysis of 1,336 patients. J Clin Oncol. 2016;34(supple; abstr 3500).

14 Appendices

Appendix 1

STAT3 antibody-9132 (Cell Signalling) Immunohistochemistry
(Performed in Institute of Cancer Sciences)

Buffer - Citrate Buffer
0.1M Citric acid (1.92g in 100ml dH_2O)
0.1M Na citrate (14.7g in 500ml dH_2O)
1.8ml citric acid, 8.2ml Na citrate, 90ml dH_2O; pH to 6.0

Dewax and rehydrate:
Dewax the slides: 2 x 3 mins in Xylene
Rehydrate: 2 x 3 mins 100% alcohol
2 mins 90% alcohol
2 mins 70% alcohol
Rinse in Water

Antigen Retrieval:
Add the slides to water bath at 96^{o}C for 20 minutes
Cool for 20 mins then wash in running water
Transfer to a staining dish with water (slides can be stored like this)
Treat with 3% H_2O_2 (13ml H_2O_2 in 387ml water) for 10 mins on a stirrer
Rinse for 1min with running H2O

Staining:
Ring sections with DAKO pen to create a barrier
Blocking solution: add 100µl of casein per 0.9 ml of TBS- buffer (200uL per slide)
Cover the section with blocking solution and incubate for 20 minutes at room temp
Blot serum from sections
Incubate in primary antibody overnight at 4^{o}C , with 200uL per slide (dilution: 1:100)
Wash 5 mins in TBSx2
Envision for 30 mins (200uL per slide)
Wash 5 mins in TBSx2
Make DAB substrate (DAKO) 1:50, 3ml of substrate buffer to 60ul of DAB chromagen.
Add as much as possible per slide. Incubate until colour develops (2-10 mins)
Wash in water 10 mins

Counterstain:
Stain in haematoxylin for 60 seconds
Rinse in running tap water
1 dip in acid alcohol
Blue with scots tap water substitute (45 sec)
Rinse in running tap water

Dehydrate and mount:
1 min 70% alcohol
1 min 90% alcohol
2 x 1 min 100% alcohol
2 x 1 min xylene
Mount in DPX

Appendix 2

pSTAT3^{Tyr705} antibody-9131 (Cell Signalling) Immunohistochemistry
(Performed in Institute of Cancer Sciences)

Buffer - Tris-EDTA Buffer pH 8 (1000mL distilled water)
0.37g Sodium EDTA
0.55g Tris in

Dewax and rehydrate:
Dewax the slides: 2 x 3 mins in Xylene
Rehydrate: 2 x 3 mins 100% alcohol
2 mins 90% alcohol
2 mins 70% alcohol
Rinse in Water

Antigen Retrieval:
Microwave on full power for 13.5 mins to warm the solution, no lid, no rubber
Add the slides and lid and microwave on full power for ~2-3 mins to bring to pressure
Microwave for 5 mins under pressure
CAREFULLY remove weight to allow steam to escape, and remove lid
Cool for 20 mins
Wash in running water
Transfer to a staining dish with water (slides can be stored like this)
Treat with 0.3% H_2O_2 (13ml H_2O_2 in 387ml water) for 10 mins on a stirrer
Rinse for 1min with running H2O

Staining:
Ring sections with DAKO pen to create a barrier
Blocking solution: add 50µl of horse serum per 0.95 ml of TBS- buffer (200uL per slide)
Cover the section with blocking solution and incubate for 20 minutes at room temp
Blot serum from sections
Incubate in primary antibody overnight at 4ºC, with 200uL per slide (dilution: 1:50)
Wash 5 mins in TBSx2
Envision for 30 mins (200uL per slide)
Wash 5 mins in TBSx2
Make DAB substrate (DAKO) 1:50, 3ml of substrate buffer to 60ul of DAB chromagen.
Add as much as possible per slide. Incubate until colour develops (2-10 mins)
Wash in water 10 mins

Counterstain:
Stain in haematoxylin for 60 seconds
Rinse in running tap water
1 dip in acid alcohol
Blue with scots tap water substitute (45 sec)
Rinse in running tap water

Dehydrate and mount:
1 min 70% alcohol
1 min 90% alcohol
2 x 1 min 100% alcohol
2 x 1 min xylene
Mount in DPX

Appendix 3

CD3 antibody 9107-S (Thermo RM) Immunohistochemistry
(Performed in University Department of Pathology using ThermoFisher Autostainer 480s)

Dewax and rehydrate:
Dewax the slides: 2 x 3 mins in Xylene
Rehydrate: 2 x 3 mins 100% alcohol
2 mins 90% alcohol
2 mins 70% alcohol
Rinse in Water

Antigen Retrieval:
Thermofisher PT Module using Thermofisher Retrieve pH9 solution
Wash in running water
Transfer to a staining dish with water (slides can be stored like this)
Treat with 0.3% H_2O_2 (13ml H_2O_2 in 387ml water) for 5 mins then rinse with TBS buffer
UV protein block for 5 mins then rinse with TBS buffer

Staining:
Ring sections with DAKO pen to create a barrier
Incubate in primary antibody overnight at room temperature, with 200uL per slide (dilution: 1:300)
Wash with TBS buffer thoroughly
Quanto Amplifier for 10 mins then rinse with TBS buffer
Quanto Polymer for 10 mins then rinse with TBS buffer
DAB Quanto Substrate for 5 mins then rinse with water

Counterstain:
Stain in haematoxylin for 3 minutes
Rinse in running tap water
1 dip in acid alcohol
Rinse in running tap water
Blue with scots tap water substitute (45 sec)
Rinse in running tap water

Dehydrate and mount:
1 min 95% alcohol
1 min 100% alcohol
3 x 5 min xylene
Mount in DPX

Appendix 4

MMR Protein (Dako UK Ltd) Immunohistochemistry
(Performed in University Department of Pathology using ThermoFisher Autostainer 480s)

Antibodies	Product code	Concentration
MLH1	M3640	1:100
MSH6	M3646	1:100
MSH2	M3639	1:50
PMS2	M3647	1:50

Dewax and rehydrate:
Dewax the slides: 2 x 3 mins in Xylene
Rehydrate: 2 x 3 mins 100% alcohol
2 mins 90% alcohol
2 mins 70% alcohol
Rinse in Water

Antigen Retrieval:
Thermofisher PT Module using Thermofisher Retrieve pH9 solution
Heated to 96^{o}C for 20 mins then coole
Wash in running water
Transfer to a staining dish with water (slides can be stored like this)
Treat with 0.3% H_2O_2 (13ml H_2O_2 in 387ml water) for 5 mins then rinse with TBS buffer
UV protein block for 5 mins then rinse with TBS buffer

Staining:
Ring sections with DAKO pen to create a barrier
Incubate in primary antibody for 20 mins at room temperature, with 200uL per slide
Wash with TBS buffer thoroughly
Quanto Amplifier for 10 mins then rinse with TBS buffer
Quanto Polymer for 10 mins then rinse with TBS buffer
DAB Quanto Substrate for 5 mins then rinse with water

Counterstain:
Stain in haematoxylin for 3 minutes
Rinse in running tap water
1 dip in acid alcohol
Rinse in running tap water
Blue with scots tap water substitute (45 sec)
Rinse in running tap water

Dehydrate and mount:
1 min 95% alcohol
1 min 100% alcohol
3 x 5 min xylene
Mount in DPX

Appendix 5

'*Tissue IA Optimiser Measured cells algorithm'* Slidepath, (Leica Biosystems)

Algorithm Preferences	Measure Stained Cells Default Preference
0=Âµm, 1=mm, 2=pixels	0
Segment Tissue from Background by Intensity	220
0=Nuclei are similar, >=1, Nuclei increasingly diverse (darkest to lightest)	2
0=Strong Nuclear Counterstaining, 2=Weak Nuclear Counterstaining	2
Values in units	37
Eliminate nuclei with area outside this range (specified in units squared)	0
Eliminate nuclei with density outside this range	0
Eliminate nuclei with nuclear area density outside this range (specified in units squared)	0
Eliminate cells with area outside this range (specified in units squared)	0
Values in units	100
Above this value pixels are identified as negative	220
Eliminate nuclei with a % below this value	10
Identify nuclei having strong/moderate/weak staining intensity	99
Above this value pixels are identified as negative	220
Eliminate areas with a % below this value	75
Identify areas having strong/moderate/weak staining intensity	160
Above this value pixels are identified as negative	220
Eliminate areas with a % below this value	75
Identify areas having strong/median/weak staining intensity	160
0 = Include All Cells, 1 = Include only Positive Cells, 2 = Include only Negative Cells	0
0 = Include All Cells, 1 = Include only Positive Cells, 2 = Include only Negative Cells	0
0 = Include All Cells, 1 = Include only Positive Cells, 2 = Include only Negative Cells	0
Default Calibration	1
Nuclear Counterstain	deconvolution-Haematoxylin
Nuclear Marker	deconvolution-DAB